GLE1 as a target for neuroprotection in Spinal Muscular Atrophy (SMA)

By

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Summary

Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder, affecting children with an incidence rate of 1 in 6000 births. Although SMA was first described a long time ago, it is still incurable. It is characterised by loss of α - motoneurons in the spinal cord and brainstem leading to reduced mobility and muscle weakness. The genetic cause is the inactivation of *SMN1* gene, thus leading to low expression of survival motor neuron (SMN). This protein is involved in mRNA splicing and β-actin mRNA transport along the axons. Although the exact molecular mechanisms have not been elucidated yet, SMN deficiency causes axonal defects and pathfinding errors in motoneurons.

A clinically similar disease is Lethal Congenital Contracture Syndrome 1 (LCCS1). This condition is inherited as autosomal recessive character and has been recently linked to mutations in *GLE1* gene. Interestingly, GLE1 is responsible for mRNA export from the nucleus to the cytoplasm. Thus, the involvement of these two proteins at different steps of the mRNA processing pathway, suggests that GLE1 might be neuroprotective in SMA.

We therefore aimed at elucidating whether GLE1 overexpression might rescue axonal defect in SMN-deficient motoneurons.

In particular, we established primary cultures from a transgenic mouse model of SMA and we modulated GLE1 expression by lentiviral vector. Preliminary, we characterised those vectors by assessing their ability to express either isoforms of GLE1 and then we investigated the axonal elongation in SMA and control motoneurons transduced with our viral vector over expressing GLE1a.

Consistently with previous reports, we observed reduced axonal length in SMA motoneurons whereas in GLE1a over expressing cells such defect was completely rescued.

Our findings support the hypothesis that GLE1 might be a neuroprotective target in SMA, although further investigations are needed to elucidate the therapeutic potential of GLE1 modulation.

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List of Abbreviations

AON - Antisense Oligonucleotide

AAV - Adeno Associated Virus

BBB - Blood Brain Barrier

BIV - Bovine Immunodeficiency Virus

BSMA - Bulbar Spinal Muscular Atrophy

EIAV - Equine Infectious Anaemia Virus

FIV - Feline Immunodeficiency Virus

HDAC - Histone Deacetylase

HIV - Human Immunodeficiency Virus

InP_6 - Inositol Hexakisphosphate

LAAHD -Lethal Arthrogryposis with anterior horn cell disease

LCCS - Lethal Congenital Contracture Syndrome

LV - Lentiviral Vector

MO - Morpholino technique

MOI - Multiplicity of Infection

NPC - Nuclear Pore Complex

NSE - Neuron Specific Enolase

PIC1n - Chloride Conductance Regulator Protein

PIPKIγ - Phosphatidylinositol-4-phophate 5-kinase type I, gamma

PRMT5 - Protein Arginine Methyltransferase 5

SAHA - Suberoylanilide hydroxamic acid

scAAV - self complementary Adeno Associated Virus

SIV - Simian Immunodeficiency Virus

SMA - Spinal Muscular Atrophy

SMARD - Spinal Muscular Atrophy with Respiratory Distress

SMN - Survival Motor Neuron

snRNA - small nuclear RNA

snRNP - small nuclear Ribonuclear Proteins

TSA - Trichostatin A

CHAPTER 1

INTRODUCTION

1.1. Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is a neurodegenerative disease for which there is no effective treatment available although it was first described by Guido Werdnig and Johann Hoffman in 1890s *(Sumner C, 2006)*. SMA is an autosomal recessive disorder with an incidence of about 1 in 6000 births and carrier frequency of 1 in every 35; therefore it is one of the leading genetic causes of infant mortality. SMA is caused by the degeneration of α – motoneurons in the spinal cord and the brain stem leading to general physical weakness and death mainly due to respiratory failure *(Lorson et al, 2010)*.

1.1.1. General pathophysiology

Denervation in SMA causes muscle atrophy predominantly in the proximal muscles i.e. the lower limbs affected more than the upper limbs which are more affected than the intercostal muscles. Facial muscles and diaphragm are generally spared in SMA affected individuals. Flaccid paralysis is considered as one of the major symptom of SMA whereas tremors in fingers, fasciculation of tongue and breathing difficulties are also reported. Survival of the child mainly depends on the respiratory support provided *(Bosboom et al, 2009).* SMA affects the motor system from the anterior horn cell to the muscle and thus, the patient has intact sensory and cognitive functions *(Montes et al, 2009).*

SMA is categorised into 4 types based on the age of onset and the severity of the phenotype. Type I, also known as Werdnig - Hoffmann disease, is the most severe form of SMA and comprises of about 50% patients diagnosed with SMA. The onset of disease is around 6 months of age whereas death is usually before 2 years. The patients suffering from SMA Type I display hypotonia, flaccid paralysis and inability to control their head movement. They generally require non-invasive respiratory

support due to their paradoxical breathing. Such type of breathing is observed because the intercostal muscles are affected whereas the diaphragm is spared. Bulbar denervation also decreases airway protection thereby increasing the risk of pneumonia. These patients are unable to sit on their own without any support (Table 1.1) *(Lunn and Wang, 2008 and Montes et al, 2009).*

Type I SMA can be further sub-divided into 3 types. The most severe Type I SMA is seen during the neonatal period and requires immediate respiratory support. Less severe form of Type I SMA patients encounter problems with feeding, handling secretions and eventually require non-invasive respiratory help. The milder form of Type I SMA patients can sit with the help of some support. The patients suffering from all three sub-classes die before 2 years of age *(Montes et al, 2009).*

Type II SMA is an intermediate form and age of disease onset is about 7-18 months of age whereas the patient usually lives for more than 2 years. Abnormal curve of the spinal cord, also known as kyphoscoliosis, is normally seen in patients suffering from Type II SMA. Hence, they require surgical or orthotic help. Similar to Type I SMA patients, this group also shows decreased airway protection, thus requiring non-invasive respiratory support. Respiratory insufficiency is a major cause of death in this group. Type II patients are able to sit unsupported but require braces to walk *(Lunn and Wang, 2008).*

Type III, also known as Kugelberg - Welander disease, is a mild form of SMA with the disease onset after 18 months of age and the life expectancy is not reduced. Most of the patients are able to walk independently whereas some might need support. During adulthood, patients often suffer muscle and joint weakness and some might develop kyphoscoliosis *(Lunn and Wang, 2008).* Type III SMA is sometimes further categorised into two types, namely; 'Type III a' where age of onset is below 3 years and 'Type III b' where age of onset is 3 years or more *(Prior et al, 2009).*

Type IV is the mildest form of SMA affecting individuals in second or third decade of life. The life of the patient is normal with mild weakness similar to those suffering from Type III SMA. Respiratory insufficiency or nutritional problems are not seen in Type IV patients *(Lunn and Wang, 2008).*

There are various forms of SMA such as spinal muscular atrophy with respiratory distress (SMARD) and bulbar and spinal muscular atrophy (BSMA), also known as Kennedy's disease *(Lunn and Wang, 2008).* Patients suffering from SMARD Type I generally do not initially experience muscular atrophy, but suffer with respiratory insufficiencies very early in their life due to diaphragm weakness *(Kaindl et al, 2008)* (Table 1.2). Conversely, patients suffering from Kennedy's disease do not show any symptoms till 40-50 years of age when they display facial muscle weakness, difficulty in speaking and fasciculation of tongue. Furthermore, this is an X – linked disorder affecting 1 in 40,000 males worldwide *(Dejager et al, 2002).*

Type of SMA	Age of Onset	Maximum function achieved	Natural age of Death
Type I	0-6 months	Cannot sit unsupported, non invasive respiratory support required	Less than 2 years
Type II	7-18 months	Can sit unsupported, but require braces to walk	More than 2 years
Type III a	18 months - 3 years	Can walk around independently without any braces, but muscles weakness present	Adult
Type III b	>3 years	Same as Type III a	Adult
Type IV	20-30 years	Can perform all functions unsupported, but mild weakness present	Adult

Table 1.1. Classification of Spinal Muscular Atrophy

Various forms of SMA	Age of Onset	Phenotype observed	Natural age of death
Spinal Muscular Atrophy with Respiratory Distress (SMARD)	0-2 months	Respiratory insufficiencies and muscle weakness later on	Less than 2 years
Bulbar and Spinal Muscular Atrophy (BSMA)	40-50 years	Facial muscle weakness, difficulty in speaking and fasciculation of tongue	Adult

Table 1.2. Various forms of Spinal Muscular Atrophy

1.1.2. Genetics of SMA

The genetic alteration causing SMA was discovered in 1995 when the gene responsible was identified and characterised. The critical region for SMA was found to be 5q11.2-13.3 by linkage analysis. After genetic mapping of the region, it was seen that Survival Motor Neuron (*SMN*) gene, which was present in duplicates, was lacking in 93% and interrupted in 5.6% of the SMA patients. Mutations were observed in this gene for the remaining patients, thereby providing evidence of its involvement in SMA *(Lefebvre et al, 1995).*

SMN gene is present in two copies in humans namely, *SMN1* (telomeric copy) and *SMN2* (centromeric copy). Both *SMN1* and *SMN2* genes have nine exons and eight introns with their sequences differing in 5 nucleotides (Figure 1.1) *(Lunn and Wang, 2008).* Among these, the most important is a nucleotide transition (C→T) at position 6 in exon 7, although this does not change the amino acid sequence *(Lefebvre et al, 1995).* It triggers alternative splicing mechanisms, which will be explained in section 1.1.2.2.

1.1.2.1. *SMN1* gene

It has been observed that approximately 94% of the SMA patients lack exon 7 of *SMN1* gene. Other 6% of them show small mutations in exon 7. Exon 7 consists of functionally critical sequence with the stop codon present near the end *(Ogino and Wilson, 2002).* It is 54 nucleotides long and is highly regulated *(Lorson et al, 2010).* Loss of *SMN1* would take place due to deletion (either exon 7 only or the whole gene) or by conversion of *SMN1* to *SMN2* gene. It has been seen that exon 8 of *SMN1* gene is sometimes physically placed close to exon 7 of *SMN2* or exon 7 of *SMN2* is flanked by intron 6 and exon 8 of *SMN1* gene *(Ogino and Wilson, 2002).* These are also known as hybrid genes. Several hypotheses like unequal recombination, gene conversion and intra-chromosomal deletions were

proposed to explain these hybrid genes *(Hahnen et al, 1996).* In 1999, 24 SMA patients were shown to lack previously recorded mutations in *SMN1* gene and were thus considered to have *SMN1*-unrelated SMA *(Wirth et al, 1999).*

1.1.2.2. *SMN2* gene

Even though mutation or loss of *SMN1* gene gives rise to SMA, it is observed that deletion in *SMN2* gene do not lead to any clinical phenotype *(Hahnen et al, 1996). SMN2* gene is only present in humans, whereas it is absent in mice and even chimpanzees. Zero to three copies of *SMN2* gene are usually present in humans. In a very rare case, a patient suffering from SMA type IV was found to have 8 copies of *SMN2* gene *(Vitali et al, 1999)*. Presence of *SMN2* gene in SMA patients modulates the severity of the disease, namely the higher copy number the less severe the phenotype. Although *SMN2* gene does not encode enough protein to compensate the loss of *SMN1* gene in patients, it does help a bit in controlling the severity of the phenotype. As mentioned before, due to the single nucleotide change in exon 7 between *SMN1* and *SMN2* genes, the *SMN2* transcript undergoes alternative splicing with skipping the exon 7. This mRNA is therefore translated into a C-terminal alternative SMN protein (SMNΔ7) protein which is rapidly degraded. Only 10% of *SMN2* pre-mRNA is properly spliced and full length SMN protein is encoded (Figure 1.1). Low SMN protein in SMA patients supports embryonic development, but is found to be inadequate for the survival of motoneurons later *(Lunn and Wang, 2008).*

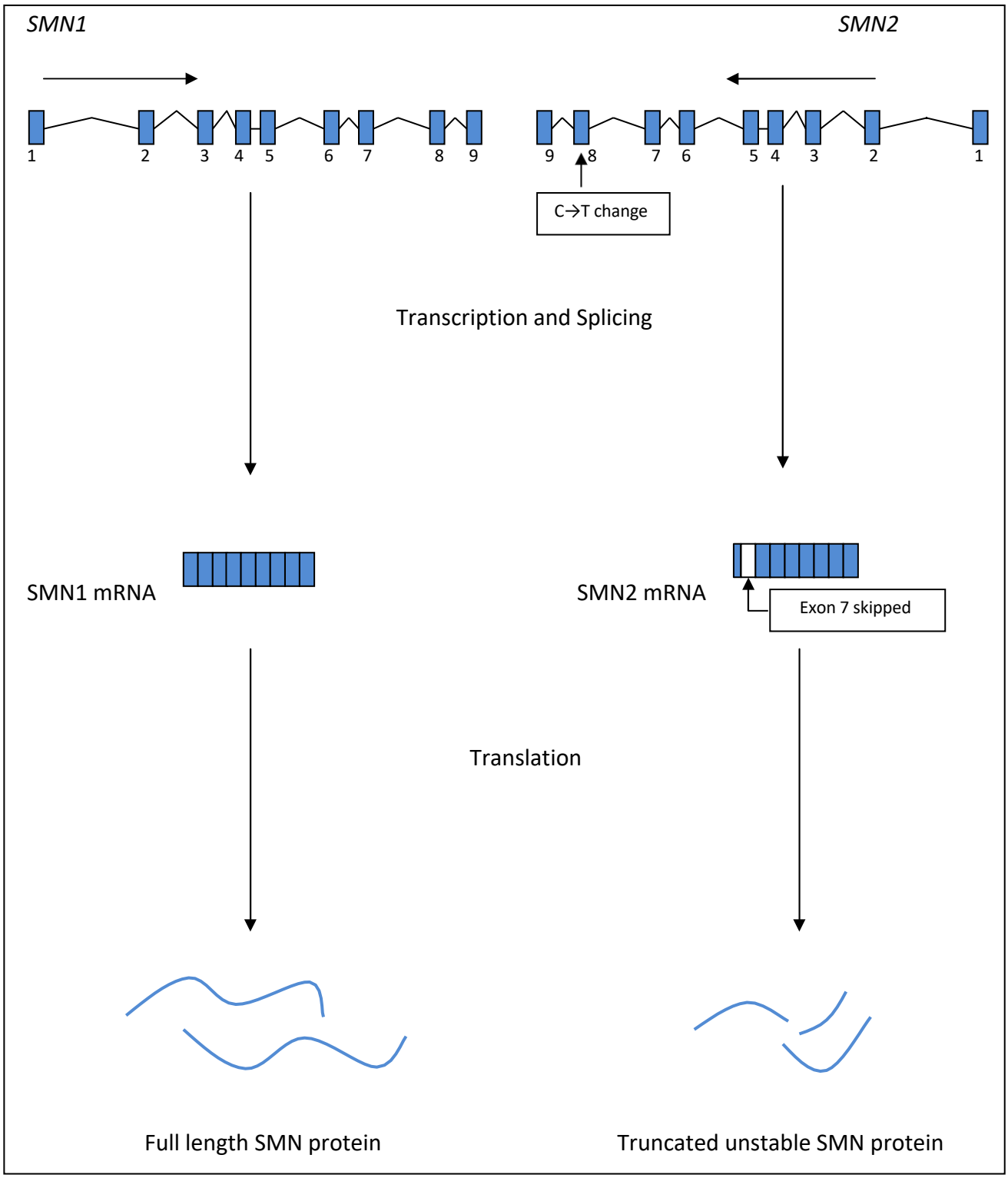


Figure 1.1. SMN1 and SMN2 gene splicing and translation

(Size of the exons and distance between exons are not to scale)

1.1.2.3. SMN protein

SMN is a 38 kD protein and is localised in the nucleus as well as in the cytoplasm. It is ubiquitously expressed but high level was observed in motoneurons of the spinal cord. SMN protein is mainly involved in small nuclear ribonucleoprotein (snRNP) assembly formation. Some of the snRNPs are essential for recognition of splice sites and removal of introns from pre-mRNA. snRNP consist of SMN complex and heptameric ring of Sm proteins. SMN complex, in turn, consist of SMN protein, UNR-interacting protein (UNRIP or STRAP) and GEMIN 2-8 proteins and its function is ATP dependent *(Burghes and Beattie, 2009).*

The snRNP assembly, required for splicing, is formed as follows –

In the cytoplasm, Sm proteins bind to Protein Arginine Methyltransferase 5 (PRMT5) complex which methylates Sm proteins and transfers them to the SMN complex. PRMT5 consists of Chloride Conductance Regulator Protein (pIC1n or ICLN) whose activity prevents Sm proteins from inappropriately binding to the snRNA. The snRNAs bind to the SMN complex and the 7-methylguanosine cap of snRNA is hyper methylated to trimethylguanosine cap thereby allowing the interaction with protein involved in nuclear import. This snRNP complex further undergoes maturation inside the nucleus making it capable of pre-mRNA splicing *(Burghes and Beattie, 2009).*

In SMA patients, SMN low level might affect the snRNP assembly thereby causing alteration in splicing of pre-mRNA. It is believed that this scarcity of snRNP assembly affects splicing of some selective genes important for motoneurons survival although SMN might have some other motoneuron specific functions that haven't been elucidated yet *(Burghes and Beattie, 2009).*

It has also been reported that SMN protein along with heterogeneous nuclear RNP - R (hnRNP - R) plays an important role in aiding the transport of β-actin mRNA through the axons of motoneurons

(Glinka et al, 2010). This localisation seems to be essential for axon initiation, growth, guidance as well as branching of the motoneuron *(Rossoll et al, 2003)*.

1.2. Lethal Congenital Contracture Syndrome (LCCS)

Lethal Congenital Contracture Syndrome (LCCS) is an autosomal recessive disorder which is responsible for prenatal death of the fetus (*Nousiainen et al, 2008).* It is mainly characterised by hydrops (accumulation of fluids in at least two fetal compartments), micrognathia, pulmonary hypoplasia, pterygia (an abnormal mass of tissue forming in the conjunctiva of the eye) and multiple joint contractures leading to total immobility. The fetus suffering from LCCS dies before the 32nd gestational week. There is a degeneration of descending tracts and anterior horn motoneurons present in the spinal cord and skeletal muscles are generally absent *(Jarvinen et al, 1998; Nousiainen et al, 2008).* The above mentioned phenotypes are similar to the fetal akinesia deformation sequence phenotype, which was formerly known as Pena-Shokeir phenotype *(Hall, 2009)*. LCCS was first seen in Finland families and has an incidence of 1 in 19,000 births *(Jarvinen et al, 1998).* LCCS is further categorised into 3 subtypes namely, LCCS1, LCCS2 and LCCS3 (Table 1.3). LCCS1 is found to be the most severe one with the phenotype same as mentioned above. The phenotype of LCCS2 is similar to that seen in LCCS1 without the presence of multiple contractures, hydrops and pterygia. However, an expanded urinary bladder is reported. In 2007, LCCS3 disorder was reported which had the same phenotype as LCCS2 without the urinary bladder defect *(Narkis et al, 2007b).*

Another disorder known as Lethal Arthrogryposis with Anterior Horn cell Disease (LAAHD) is characterised by fetal akinesia, arthrogryposis and loss of motoneurons but it found to be milder than LCCS. The child survives delivery but die early due to respiratory insufficiencies *(Nousiainen et al, 2008).*

Motor neuron degeneration in all these disorders leads to a similar but more severe phenotype as compared to SMA.

Types of LCCS	Phenotype observed
LCCS1	Hydrops, micrognathia, pulmonary hypoplasia, pterygia and multiple joint contractures
LCCS2	Micrognathia, pulmonary hypoplasia and extended urinary bladder
LCCS3	Micrognathia, pulmonary hypoplasia without the extended urinary bladder

Table 1.3. Various sub-types of Lethal Congenital Contracture Syndrome (LCCS)

1.2.1. Genetics of LCCS

LCCS1 arises due to loss or mutation in a gene known as *GLE1* and encodes a protein responsible for mRNA export from nucleus to the cytoplasm. With the help of linkage analysis done in Finnish families, the mutations were mapped in the region of chromosome 9q34. Various mutations were observed after sequencing. The DNA of an individual who survived after delivery was sequenced and was diagnosed with SMA type I disorder but no deletions or mutations were observed in *SMN* gene *(Nousiainen et al, 2008).*

An A→G substitution in exon 11 of *ERBB3* gene was identified in all the Israeli-Bedouin kindreds who showed symptoms similar to LCCS2. The locus was mapped in the region of chromosome 12q13. The mutations in *ERBB3* gene is said to be occurred due to abnormal splicing leading to a truncated non-functional protein. HER3, the protein encoded by *ERBB3*, is an activator of phosphatidyl inositol pathway *(Narkis et al, 2007a).*

The locus of LCCS3 was mapped to the region of chromosome 19q13. A homozygous mutation (G→A) was observed in exon 7 of *PIP5K1C* gene of all the individuals suffering from LCCS3 in the family. Phosphatidylinositol-4-phophate 5-kinase type I, gamma (PIPKIγ) is encoded by *PIP5K1C* gene and is one of the enzymes essential in the phosphatidyl inositol pathway (Narkis et al, 2007b).

1.2.1.1. *GLE1* structure and function

GLE1 gene comprises of 16 exons and the protein encoded is cytoplasmic but it can also cluster on the nuclear envelope and interact with the elements of the Nuclear Pore Complex (NPC). There is a 5' cryptic splice site present in exon 14 which gives rise to the two isoforms of GLE1 in humans. hGle1a and hGle1b differ only in their COOH terminus, being hGle1a (659 amino acids) shorter and

much less expressed as compared to hGle1b (698 amino acids) *(Nousiainen et al, 2008; Kendirgi et al, 2003)* (Figure 1.2)

GLE1 plays an important role in mRNA export from the nucleus to the cytoplasm. GLE1, as described in yeast, is present only in the nuclear rim whereas in humans, it shuttles between nucleus and the cytoplasm *(Alcazar-Roman et al, 2010).* Interestingly, GLE1 binds to Inositol hexakisphosphate ($InsP_6$) which is obtained from the phosphatidyl inositol pathway *(Nousiainen et al, 2008).* This evidence, along with the genetics of LCCS2 or LCCS3 (see previous paragraph), support the hypothesis that alterations in mRNA transport might have detrimental effect on motoneuron viability.

The entire mRNA export mechanism can be summarised as follows. Pre-mRNA undergoes splicing and 5' capping and 3' end processing while in the nucleus. Several proteins such as Mex67/Mtr2 and Yra1 bind to the mRNA and facilitate its movement to the NPC. The DEAD-box protein Dbp5 then interacts with Yra1, which is essential for mRNA processing, and shuttles from the nucleus to the cytoplasm. Once Dbp5 comes in contact with GLE1-$InsP_6$ complex, the ATPase activity of Dbp5 is activated. This stimulates the release of Mex67/Mtr2 from the mRNA thus, inhibiting re-import *(Hurt and Silver, 2008).*

Wierich et al stated that GLE1 was essential for activation of ATPase activity of Dbp5 whereas $InsP_6$ was required for modulation of GLE1-Dbp5 complex only in the presence of mRNA. According to this report, Dbp5 is present at NPC (only in Yeast) and interacts with Nup159, Nup42 and Gfd1. Nup159 helps in recruiting Dbp5 to the cytoplasmic side of the NPC. Moreover, Nup159-$InsP_6$ complex which is present at the NPC helps in stabilizing Dbp5-GLE1-mRNA complex *(Wierich et al, 2006).*

Along with the function of mRNA export, it was observed that GLE1 also plays a role in initiation as well as termination of translation. Genetic and physical interaction was observed between GLE1 and subunits of initiation factor eIF3, thus indicating a role of GLE1 in initiation of translation. Defects in

translational initiation were found in *GLE1* mutant yeast. Moreover, genetic and physical interaction was seen between GLE1 and termination factor Sup45/eRF1. It is predicted that IP_6/GLE1 plays a role in termination of translation by the activation of Dbp5 *(Bolger et al, 2008).*

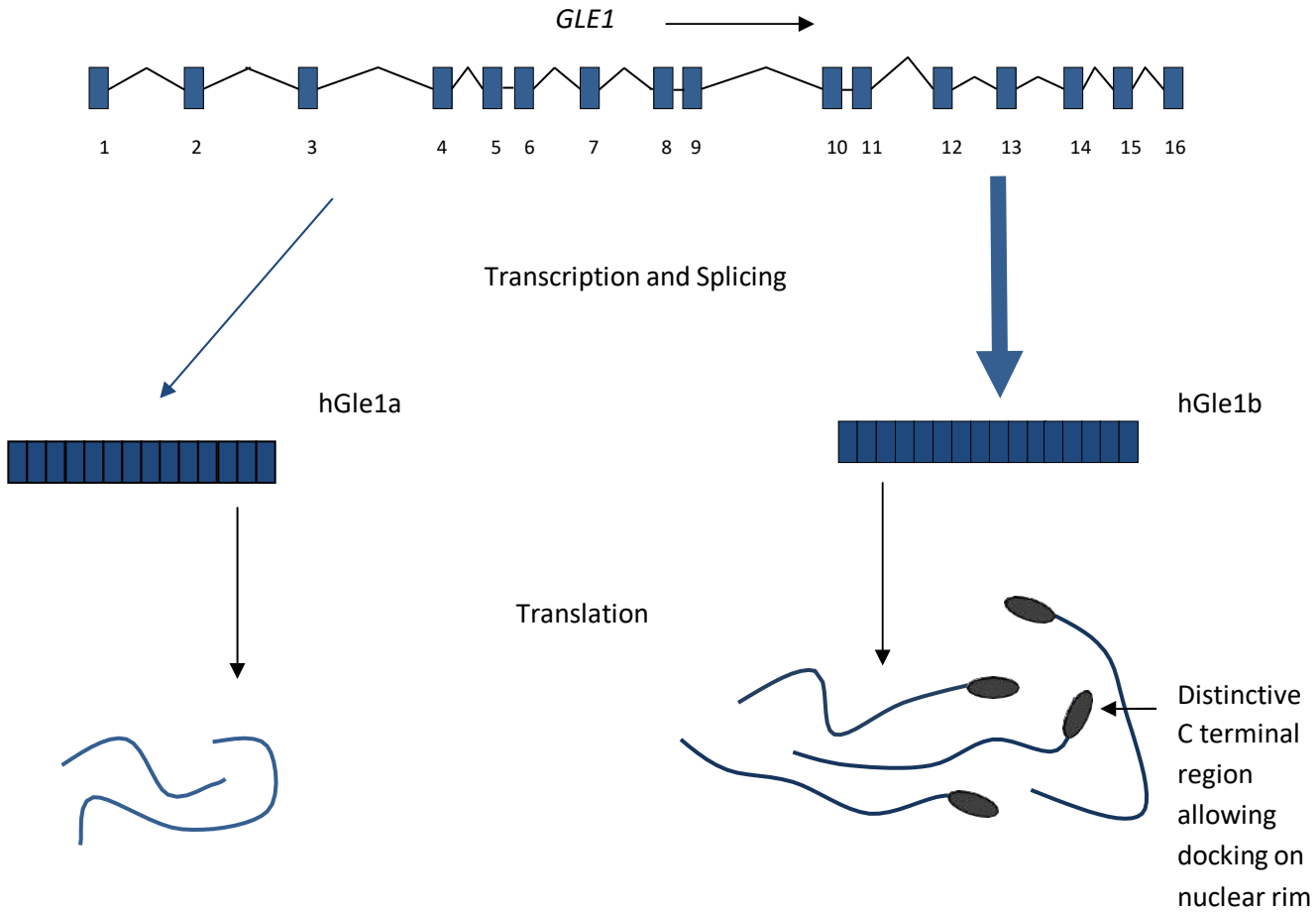


Figure 1.2. Gene representation of GLE1 isoforms

(Size of the exons and distance between exons are not to scale)

1.3. Animal Models used for SMA

Mouse and Zebrafish models are generally used to depict human diseases. Drosophila was also used, but some people do not consider them as an effective model.

1.3.1. Mouse Models

Mouse models have been used since the last decade to study different aspects of SMA. Mice harbour only one copy of *Smn* gene which is considered homologous to *SMN1* gene in humans. Interestingly, knockout of the gene leads to embryonic lethality *(Schrank et al, 1997; Monani et al, 2000a).* In order to generate a mouse model of SMA, Frugier et al in 2000, generated a transgenic mouse in which the Cre-LoxP system allows conditional deletion of exon 7 of *Smn* gene. In particular, they crossed these mice with a strain expressing Cre recombinase under the control of Neuron Specific Enolase (NSE) promoter to specifically regulate and knock out exon 7 in neurons. The offspring of this crossing displayed an average survival age of 25 days, thereby demonstrating that SMN deficiency in motoneurons is sufficient to trigger the pathological phenotype *(Frugier et al, 2000)*.

Other approach was to introduce human *SMN2* gene copies in $Smn^{-/-}$ mice. *SMN2* gene including its promoter was cut using *Bam*HI and then microinjected into mouse oocytes *(Schrank et al, 1997).* SMN expression from *SMN2* rescued the embryonic lethality and mice developed SMA-like symptoms whose severity was higher in animals carrying low copy number *(Monani et al, 2000b).*

Such a $Smn^{-/-}$; $SMN2^{+/+}$ mouse model was used to study whether the lack of Smn protein is responsible at neurodevelopmental or neuromaintenance stage. In particular, it was seen that Smn

deficiency led to an increase in cell death particularly in the telencephalon region and some morphological defects in spinal nerves of mutant embryos.

Another mouse model was generated to determine the role of SMNΔ7 in SMA. A study indicated that the level of SMNΔ7 was higher in patients suffering from Type I and Type II when compared to that observed in Type III patients *(Gavrilov et al, 1998).* Thus, to demonstrate whether the presence of SMNΔ7 is beneficial or detrimental in SMA, a *SMN* cDNA lacking exon 7 was introduced in the severe model of SMA described previously thereby, generating $Smn^{-/-}$, $Smn^{+/+}$ and $SMN\Delta7^{+/+}$ mice *(Le et al, 2005)*. It was observed that the life span of this mice model was around 13 days as compared to 5 days of $Smn^{-/-}$,$SMN2^{+/+}$ mice model. This proved that the presence of SMNΔ7 protein is not detrimental in SMA mice *(Le et al, 2005).* This mouse model is also considered useful to screen drugs to either activate *SMN* promoter activity or to increase the amount of full length SMN protein (explained in details in section 1.4). SMNΔ7 mice model was used for the experiments described in the thesis.

1.3.2. Zebrafish model

Zebrafish is considered to be one of the easiest animal models for studying human diseases. Morpholino antisense oligonucleotide (MO) technology was used to knockdown *Smn* gene in Zebrafish. Around 450 embryos out of 580 survived after *Smn* MO injection but did not show any defects. However, Smn protein was found to be essential for the development of motoneurons. Shorter axons and dose-dependent branching of axons were observed in *Smn* MO injected embryos as compared to normal embryo *(McWhorter et al, 2003).* Remarkably, it was also observed that injecting *Smn* MO into the embryos neither affected other neuronal cell types nor played any role in muscle development. Finally, the axonal branching was seen to be rescued when human *SMN* mRNA

was injected along with the *Smn* MO into the embryos, thus confirming that the phenotype aroused is due to the lack of Smn protein *(McWhorter et al, 2003).*

1.4. Therapeutic strategies

As for all neurodegenerative disorders, SMA is still incurable and only supportive therapy is currently provided. However, several approaches are currently investigated aiming either at restoring SMN expression or at supporting and protecting the surviving cells. Some of them are listed below -

1.4.1. Enhancing *SMN2* gene transcription

When 3 families consisting of individuals suffering from SMA (type II and III) were studied, it was seen that they had 2-4 copies of *SMN2* gene *(Jedrzejowska et al, 2008)*. There was a homozygous absence of *SMN1* gene in SMA affected as well as asymptomatic patients. There was one individual in each of the family lacking *SMN1* gene but was unaffected. Those individuals consisted of 4-5 copies of *SMN2* gene. Normal amount of SMN protein was seen in their fibroblast when checked *(Jedrzejowska et al, 2008).* It was also observed that when 2 copies of cDNA encoding *SMN2* gene were inserted in $Smn^{-/-}$ mice, they showed prolonged survival. The $Smn^{-/-}$; $SMN2^{+/+}$ transgenic mice survived almost 3 weeks after birth *(Monani et al, 2000b).* Thus, targeting SMN protein expression from *SMN2* gene has become one of the most promising therapeutic strategies.

In order to identify small drugs interacting with full length *SMN* mRNA obtained from *SMN2* gene, a high throughput screening assay was performed on over 555,000 compounds. Two compounds namely, Trichostatin A (TSA) and sodium butyrate were found to increase the SMN protein level in

patient derived cells *(Jarecki et al, 2005)* and their potential benefit was validated in the severe mouse model of SMA. In particular, chronic treatment with TSA in *Smn*$^{-/-}$;*SMN2*$^{+/+}$ transgenic mice led to increase in SMN protein level by 1.5 to 2 fold as well as increase in *SMN2* derived transcript *(Avila et al, 2007).* Oral administration of Sodium butyrate through drinking water, extended *Smn*$^{-/-}$; *SMN2*$^{+/+}$ transgenic mice survival by 4-5 days over the untreated littermates *(Chang et al, 2001).* These compounds act as Histone Deacetylase (HDAC) inhibitors thus up-regulating approximately 2% of the total genes *(Sendtner, 2010)*. Another HDAC inhibitor, Suberoylanilide hydroxamic acid (SAHA), was injected into *Smn*$^{-/-}$; *SMN2*$^{+/+}$ transgenic mice. The treated mice showed more level of SMN protein, reduced degeneration of motoneurons and 30% increase in lifespan as compared to the untreated ones *(Reissland et al, 2010).*

Valproic acid, commonly used as antiepileptic drug, is also a HDAC inhibitor and it was also seen to increase the SMN protein in SMA fibroblast cells by either activating the *SMN* promoter or by preventing the exon 7 skipping of *SMN2* during transcription *(Sumner et al, 2003).* Furthermore, it was found to normalise motor function, reduce motoneuron death as well as increase spinal SMN protein level in *Smn*$^{-/-}$; *SMN2*$^{+/+}$ mice *(Tsai et al, 2006).*

Three compounds of 2, 4-diaminoquinazoline family were given orally to SMNΔ7 mice. One of them, D156844, significantly increased the lifespan of the mice by 21-30% as compared to the untreated animals. It also increases the overall expression of SMN in SMNΔ7 mice *(Butchbach et al, 2010).*

1.4.2. Modulating *SMN2* splicing

Although antisense oligonucleotides (AON) are generally used for inhibiting the expression of a particular gene, they can also be used to modulate pre-mRNA splicing at specific sites by silencing

the splicing sites. Injection of AON targeting *SMN2* exon 7 in *Smn*$^{-/-}$; *SMN2*$^{+/+}$ transgenic mice, increased exon inclusion in liver, kidney and in muscles as compared to the untreated mice was observed. However, there was no change in SMN protein level in the spinal cord as AON do not cross the Blood Brain Barrier (BBB). Therefore, efficient tools to deliver AON across the BBB are needed to demonstrate the potential therapeutic benefits of this approach in SMA *(Hua et al, 2008).*

In another experiment, it was seen that AON specific to silence a splice site, enhanced exon 7 inclusion thereby increase the amount of SMN full length protein. They also showed that despite hybridizing an exon, AON do not interfere with the mRNA export or translation. Thus, predicting the use of antisense oligonucleotide for further testing in animal models *(Hua et al, 2007).*

A compound, hydroxyurea, was tested for its capability in modulating *SMN2* splicing pattern to give full length SMN protein *(Grzeschik et al, 2005).* Lymphoblastoid cells derived from SMA patients were subject to various concentration of hydroxyurea thereby observing an increased ratio between the full length *SMN* mRNA to truncated *SMN* mRNA level. Furthermore, a higher dose of hydroxyurea showed significant increase in not only the ratio but also in SMN protein level. Although the exact mechanism of hydroxyurea is still unknown, it is believed that the increase was due to the change in *SMN2* splicing pattern to give full length SMN protein *(Grzeschik et al, 2005).*

1.4.3. Trans-splicing system

A trans-splicing system consists of co-expressing an antisense RNA which would block the splice product of *SMN2* pre-mRNA and a *SMN2* trans-splicing RNA. Plasmids expressing these RNAs were injected into the intracerebral ventricular space of the *Smn*$^{-/-}$; *SMN2*$^{+/+}$ neonate mice and a

significant 70% increase in the survival was observed as compared to the untreated ones *(Coady and Lorson, 2010).*

1.4.4. Gene therapy

Viral vectors can be considered as one of the easiest tools through which gene therapy can be used as a therapeutic strategy. Depending on the experiment design, viral vectors can be used for various purposes such as knock down of genes, to restore the protein level by gene replacement or to deliver neurotrophic factors *(Davidson and Breakefield, 2003).* Furthermore, these vectors may be used also to generate *in vivo* models of human diseases. Various types of viral vectors are used for research purposes. Some of them are listed below -

1.4.4.1. Adeno-Associated Virus (AAV)

AAV are single strand DNA viruses belonging to the family of Parvoviridae. They are widely used as they can transduce both dividing and non-dividing cells such as neurons with a high efficiency and they are found to shows little or no immunogenicity. Although, they generally do not integrate into the host genome, their gene expression is stable. They exhibit a small cloning capacity (insert size) i.e. 4.5 kb. It is also seen that they do not transduce all the cell types with the same efficiency *(Davidson and Breakefield, 2003).*

Although AAV normally package single stranded genomes, they could be modified to package either two copies or dimeric inverted repeats of small genomes thus giving rise to Self-complementary adeno-associated virus (scAAV) *(McCarty et al, 2001)*. scAAV9 expressing *SMN* were injected

intravenously into neonatal SMNΔ7 mice to study the therapeutic effect of SMN delivery at different time points, namely at postnatal day 1, day 5 and day 10. It was seen that the maximum rescue of the phenotype was observed when the virus was injected at the earliest. The scAAV9 was capable of transducing approximately 60% of the motoneurons *(Foust et al, 2010).*

1.4.4.2. Lentiviral Vectors (LV)

Lentiviruses are RNA viruses belonging to the family of Retroviridae displaying a larger cloning capacity i.e. 8-10 kb in size. The most important advantage of LVs is their capacity to transduce dividing as well as non-dividing cells (e.g. neurons) very efficiently. They do not generate any inflammatory response in the target cells. However, they integrate the genes into the genome of target cells leading to a longer, stable expression, at the same time raising doubts about their safety as they are natural pathogens (e.g. HIV-based) and the disease they can cause would be incurable. LVs can be classified on the basis of their natural host into viruses infecting either primates [(Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency Virus (SIV)] or non-primates [Equine Infectious Anemia Virus (EIAV), Feline Immunodeficiency virus (FIV) and Bovine Immunodeficiency virus (BIV)] *(Azzouz et al, 2004a; Nanou and Azzouz, 2009).*

Interestingly, EIAV-based LVs display retrograde transport upon intramuscular injection, thereby transducing motoneurons *(Azzouz et al, 2004b)*. Taking advantage of this feature, an EIAV vector was designed to overexpress SMN and was firstly validated in fibroblasts obtained from SMA type I patient. Then the vector was injected into various muscles of SMNΔ7 mice model thus transducing almost 70% of the motoneurons. Therefore, SMN level was restored in the motoneurons and the lifespan was extended by 3 and 5 days more as compared to treated with a control virus and untreated respectively *(Azzouz et al, 2004b).*

1.4.5. Stem Cell therapy

A promising approach to restore motor function in SMA relies on the potential of stem cell to differentiate into different cell types thereby generating new motoneurons to substitute those lost in the disease process. In particular, when embryonic stem cells derived neural stem cells were injected in SMNΔ7 mice models, they differentiated into motoneurons and migrated to appropriate regions and increased the lifespan of the treated mice by 8 days as compared to the untreated SMNΔ7 mice *(Corti et al, 2010).*

1.5. Hypothesis and Aims of the project

As discussed above, the function of SMN is mainly involved in snRNP assembly which is essential for proper splicing of pre-mRNA whereas the best characterised function of GLE1 is to mediate mRNA export from the nucleus to the cytoplasm for translation into proteins. Therefore, SMN and GLE1 act at different stages of a single pathway whose steps are highly coupled *(Carmody and Wente, 2009; Kohler and Hurt, 2007).* SMN also functions by aiding the translocation of β-actin mRNA through the axons upon interaction with hnRNP - R *(Glinka et al, 2010).*

Moreover, the diseases which arise due to the absence of both the proteins (SMN and GLE1) share certain pathological similarities. The phenotype observed in patients suffering from SMA or LCCS1 are quite similar, but more severe in case of LCCS1. Thus, the general hypothesis would be to test whether the loss of one protein (SMN) can be compensated by the overexpression of the other protein (GLE1).

Preliminary data obtained by Rengina Marketou within the host laboratory (unpublished data) demonstrated axonal defects in cultured motoneurons depleted of *Smn* with short hairpin RNA (shRNA) whereas similar defects were observed in motoneurons purified from a mouse model of

severe SMA. Firstly we hypothesized that motoneuron deficits were also a characteristic of SMNΔ7 mice.

Then, we hypothesised that overexpression of either GLE1 alone or GLE1 and SMN compensates the absence of SMN by significantly rescuing motoneurons deficits.

If GLE1 is not able to significantly rescue the motoneuron defects due to the lack of SMN, it would be interesting to check whether synergistic effect of SMN and GLE1 would rescue the phenotype.

1.5.1. Aims of the project

We therefore plan to (1) assess whether motor neurons purified from SMNΔ7 mice display abnormalities in axonal morphology and (2) test whether overexpression of GLE1 (either isoforms hGle1a or hGle1b) modulates axonal growth in purified motoneurons obtained from SMNΔ7 mice model. The axonal length measurement would be done and compared to controls (i.e. motoneurons obtained from carrier littermate embryos and untransduced cells from *Smn*$^{-/-}$ embryos).

CHAPTER 2

MATERIALS AND METHODS

2.1. Mice

All animal procedures were carried out in accordance with the Home Office regulations. *Smn* $^{+/-}$; *SMN2*$^{+/+}$ and SMNΔ7$^{+/+}$ mice *(SMNΔ7 mice, Le et al, 2005)* were purchased from the Jackson Laboratories and a colony was established in our animal facility. Breedings between *Smn*$^{+/-}$; *SMN2*$^{+/+}$ and SMNΔ7$^{+/+}$ mice were allowed and their date were recorded. The female was culled by cervical dislocation at 13.5 days of pregnancy and the embryos were collected. Due to the breeding strategy, each embryo had to be processed independently and its genotype was determine as follows -

2.1.1. Tail digestion

A small biopsy of the tail was collected from the each embryo and incubated with 30µl of DNA extraction buffer (Epicentre Biotechnologies) for 30 minutes at 62°C followed by 5minutes at 98°C to halt the reaction.

2.1.2. Polymerase Chain Reaction (PCR)

PCR mix was prepared as described in table 2.1 and 2.2. For each reaction, a negative control and 3 positive controls, namely *Smn* $^{+/+}$, *Smn* $^{+/-}$, *Smn* $^{-/-}$ were included. The thermocycler was programmed as follows -

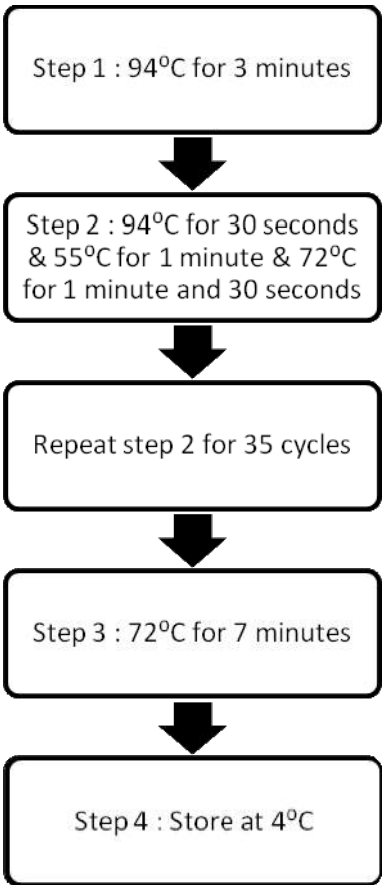


Figure 2.1. Thermocycler program for PCR

Reagents	Volume to be added (µl)
dH_2O	10.08
Smn forward primer 50*µM*	0.67
Smn reverse primer (Wild type) 50*µM*	0.5
Smn reverse primer (Mutant) 50*µM*	0.25
PCR master mix	12.5
Template	1.0

Table 2.1. Concentration of reagents to be added for each sample for genotyping

	Primers
Smn forward primer	CTC CGG GAT ATT GGG ATT G
Smn reverse primer (Wild type)	TTT CTT CTG GCT GTG CCT TT
Smn reverse primer (Mutant)	GGT AAC GCC AGG GTT TTC C

Table 2.2. Primer sequences used for genotyping

The samples were then loaded on a 1.5% agarose/TAE (Trizma base 0.04M, acetic acid 0.02M, EDTA 1mM) gel supplemented with 0.5µg/µl of Ethidium Bromide (EtBr), a reagent that binds to DNA increasing its fluorescence, thereby allowing DNA visualization upon UV irradiation. Size-dependent separation of the amplicons was obtained by applying an electric field at 100V for 30 minutes. After the electrophoresis the gel was irradiated with UV light and a picture was taken using the software Alpha-Imager. The expected band for the wild type allele was around 800 base pairs (bp) whereas that for the mutant was around 500 bp (Appendix Figure 6.1)

2.2. Motoneuron culture

Purified motoneurons were provided by Dr. Ke Ning. 12 well plates and 35mm dishes with 4 x 10mm rings were coated with 5% Poly-L-ornithine (PORN, Sigma, P -8638) in 0.15M Borate buffer, pH 8.35 and incubated overnight at 4°C. The plates and dishes were washed twice with Hank's Buffered Salt Solution (HBSS) from GIBCO, to remove the PORN buffer and then coated with laminin (stock 0.71mg/ml in 50mM Tris-HCL, pH 7.4, diluted to 15µg/ml in HBSS with Phenolred, Invitrogen 23017-015) and incubated for 1-2 hours at 37^{o}C (Colombi et al, 2013).

SMNΔ7 mice were killed by cervical dislocation and embryos were extracted. The spinal cords were isolated, their meningis removed and tissues were incubated in 0.1 % trypsin (Worthington LS003707) in HBSS with Phenolred for 15 minutes at 37^{o}C. Tails were also collected for genotyping as described. The reaction was stopped using 0.1% trypsin inhibitor (Sigma T6522-500) and the spinal cords were transferred to Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza, BE17-512F) and homogenised. The suspension was added to dish precoated with p75 antibody (Abcam, ab8877) diluted 1:5000 in Tris buffer at pH 9.5 and incubated for 30-40 minutes. NGFp75 receptors are specifically expressed on cell membranes of motoneurons that therefore bind to the plate whereas

other cell populations are washed off when rinsed with neurobasal media (GIBCO, Invitrogen 21103049) with beta-mercaptoethanol. The motoneurons are detached using 0.2ml of depolarising solution (30mM KCl and 0.8% NaCl) for each well of 12-well plate and resuspended in 0.8ml full culture medium (neurobasal media supplemented with with 1% glutamax - 1 (GIBCO, Invitrogen 35050-038), 2% horse serum donor (Linaris, SHD3250YK), 1x B27 (GIBCO, Invitrogen 17504-044), BDNF (Sigma B3795) and beta-mercaptoethanol). Cells were counted using haemocytometer and were plated at a density of 2000 cells per well in each 10mm ring and 60,000-80,000 cells per well in 12 well plate. Cells were incubated in low amount of media for 30-40 minutes to allow cell adhesion and then media was added. The cells were incubated at 37°C with 5% CO_2 for 7 days. The medium was changed every alternate day i.e. on the 3rd day and 5th day after plating. 1ml of the old media was taken out and 1ml of fresh media was added to the wells.

2.3. Generation of Lentiviral Vector

2.3.1. Cell Culture

Human Embryonic Kidney cells line (HEK293T) was used for the production of Lentiviral vectors. HEK293T cells were cultured in Dulbecco's Minimum Essential Media (DMEM) (Lonza BE12-741F) with 10% heat inactivated Fetal Calf Serum (FCS, Biosera Lot 50566351810) and grown at 37°C and in 5%CO_2 with 95% humidified air atmosphere. Twice a week they were subcultivated 1:8. Briefly, the medium was removed and the cells were incubated for a few minutes in 2 ml of trypsin supplemented with 0.5mM EDTA in PBS. When the cells detached into a single cell suspension, the enzymatic activity was finally stopped by adding an excess of culturing medium.

2.3.2. Viral production

Lentiviral vectors were prepared by transient cotransfection using a calcium phosphate method (Deglon et al, 2000, Mahajani et al, 2019). In particular, the cells are counted and plated at 3.6-3.8 x 10^6 cells per 10cm dish, in the afternoon prior to transfection, as to obtain about 70% confluency the following morning.The transfection mixture was prepared by adding 13µg of pCMVΔR8.92, 3µg of pRSV-Rev, 13µg pMD.G and 13µg of either SIN-W-PGK-GLE1a (LV-GLE1a) or SIN-W-PGK-GLE1b (LV-GLE1b) in 0.25M

$CaCl_2$. The function of each plasmid is mentioned in table 2.3. This DNA mix was then added drop-wise to an equal volume of 2x HBS buffer (5M NaCl, 11.9g of HEPES and 269 mg of Na_2HPO_4) pH 7.1. The calcium-phosphate was allowed to form a thin precipitated for 10-15 minutes at room temperature and finally 1ml was carefully added drop-wise to each dish. After 6-7 hours of incubation, the media was removed and the cells were incubated with fresh media (10ml) for three days.

The supernatant containing the virus was harvested 73 hours post transfection and centrifuged at 1000rpm for 10 minutes to precipitate cell debris. The viral suspension was then filtered through a 0.45µm syringe filter and finally ultracentrifuged at 4°C at 19,000 rpm for 90 minutes. The supernatant was discarded and the viral pellet was resuspended in PBS containing 1% Bovine Serum Albumin (BSA) from Sigma, divided into 20µl aliquots and stored at -80°C where lentiviral vectors can be kept for at least 1 year with no significant loss of titre.

	Plasmid Vector	Function
1.	pCMVΔR8.92	Packaging vector – It encodes all the viral proteins needed for viral assembly and activity in target cells.
2.	pRSV-Rev	Plasmid encoding the rev protein of HIV-1.
3.	pMD.G	Plasmid encoding the vesicular stomatitis virus G envelope glycoprotein.
4.	SIN-W-PGK	Transfer plasmid containing an internal cassette to drive the expression of the gene of interest.

Table 2.3. Functions of plasmids used for the production of Lentiviral vectors

2.4. Viral titration by ELISA

Viral titration was performed by Enzyme Linked Immunosorbent Assay (ELISA) targeting HIV-1 p24 antigen (ZMC No. 0801111, ZeptoMetrix Corporation) according to the protocol provided. Briefly, the microwells were coated with a monoclonal antibody specific to p24 *gag* gene protein of HIV-1. When the samples were added, the viral antigen bound onto the immobilized monoclonal antibody and the captured antigen was further recognised by titred human anti-HIV - 1 antibody conjugated with biotin. Finally, streptavidin peroxidase and its substrate solution were added and the reaction was stopped by acidification thereby obtaining a yellow colour whose optical density was proportional to the amount of HIV-1 p24 antigen present.

2.4.1. Calculating viral titre

The p24 ELISA assay provided us the amount of viral protein and we therefore needed to convert it into number of viral particles. The kit specified that 1ng/ml of p24 corresponds to 1.2 x 10^4 Transducing Units (TU) per ml. Ideally, the viral titre should be in the range of 1 x 10^6 to 1 x 10^9.

2.5. Transduction

Motoneurons obtained from mice embryos were transduced using Lentiviral vectors expressing either GLE1a or GLE1b, the next day after plating. As transducing control, LV-GFP was also made to transduce HEK293T in one experiment (Giacomini et al, 2016, Mahajani et al, 2017). The amount of virus to be added was calculated using the formula,

$$\text{Amount of Virus to be added } (\mu l) = \frac{\text{Number of cells per well x MOI}}{\text{Viral titre}}$$

Where MOI is multiplicity of infection, the ratio between the number of viral particles and the number of cells

The cells were incubated at 37°C for 6 days, and then they were harvested and lysed on 7th day after plating.

In particular, the media was carefully removed from each well to avoid detaching the cells and each well is washed with DPBS. Lysis was performed with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% of Igpal and 0.1% SDS) supplemented with Protease Inhibitor Cocktail (PIC) from Roche (Cat. No. 11836170001) incubating the plate on ice for 45 minutes. Then a cell scraper was used to collect the lysates that finally centrifuged at 10000 rpm for 10 minutes. The supernatant was then collected and stored at -20^{o}C until further use.

A similar procedure was also performed to harvest the HEK293T cells that are loosely adherent and therefore the scraping was unnecessary. DPBS was used to detach the cells by pipetting. The cell suspension was spun down (500g, 5minutes) and the cell pellet was lysed and processed as described previously.

2.6. mRNA Quantification

2.6.1. Total RNA isolation

Total RNA extraction is performed using Promega SV Total RNA Isolation kit (Z3100). The procedure was carried out as mentioned in the protocol provided with the kit. A short representation of the protocol is described below -

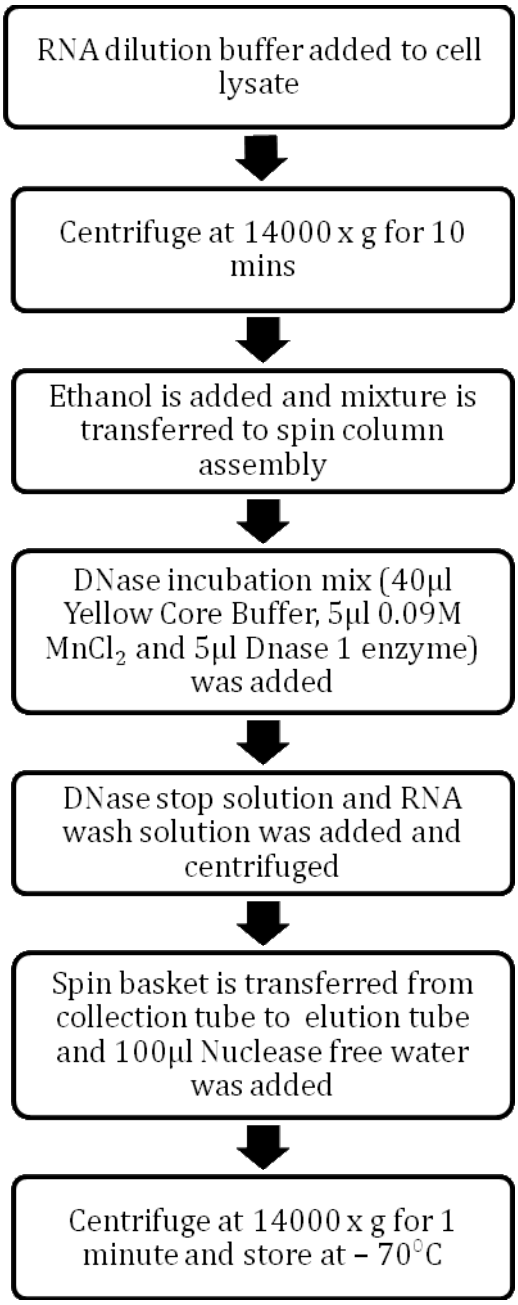


Figure 2.2. Short protocol for Total RNA extraction

2.6.2. Retro - transcription

As cDNA is more stable than mRNA, retro transcription was done with the help of SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) as described in the kit. Briefly, RNA with random hexamers and dNTPs were mixed and incubated at 65^{o}C for 5 minutes then chilled on ice. Then the samples were supplemented with cDNA synthesis mix and incubated at 50^{o}C for 50 minutes followed by 5 minutes at 85^{o}C. RNase H was finally added and the reaction was performed at 37^{o}C for 20 minutes to remove RNA template from the cDNA:RNA hybrid molecule by digestion.

2.6.3. Real Time PCR (RT-PCR)

m-RNA quantification was done on the retrotranscript using a SYBR green protocol. Briefly, two sets of tubes were prepared, one for the transcript of interest and the other for a housekeeping transcript. Nuclease free water, forward primer and reverse primer (table 2.5), template and SYBR green mixture (ABI) were mixed as described in table 2.4. The plate was then loaded in the RT-PCR (Stratagene) machine and the following program was performed –

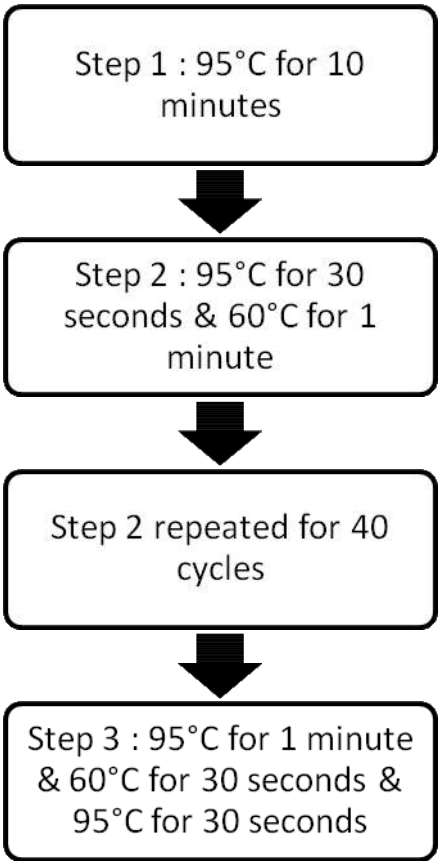


Figure 2.3. Protocol for RT-PCR

	Nuclease free water (µl)	**Forward primer *5µM* (µl)**	**Reverse primer *5µM* (µl)**	**Template 4ng/*µl* (µl)**	**SYBR green mix (µl)**
GLE1a	14.7	8.4	8.4	3.5	35.0
GLE1b	14.7	8.4	8.4	3.5	35.0
human β-actin	23.1	4.2	4.2	3.5	35.0

Table 2.4. Concentrations of reagents to be added to make RT-PCR mix

Sample	**Forward Primer**	**Reverse Primer**
GLE1a	CTATTACCTGCTGGTTTTGATTCAAG	AATATCAACGAGAGGGACTGGAGTA
GLE1b	TCTCCTCCCTTGTCTCTAGTGTCTTT	GGTAGGTGGGTGCAAATGGT
human β-actin	CAATGAGCGCGGTTCCGATACC	TCAACGTCACACTTCATGATGGA

Table 2.5. List of forward and reverse primers for RT-PCR mix

2.6.4. mRNA quantification using ΔΔCt method

Estimation of the differential presence of the mRNA of specific transcripts (ST) in experimental samples versus control condition, namely untreated cells was calculated using the following equation -

Relative quantity = $2^{-(\Delta\Delta Ct)}$

Where,

$$\Delta\Delta Ct = (Ct_{ST} - Ct_{HK})_{experimental\ sample} - (Ct_{ST} - Ct_{HK})_{control}$$

And Ct is the cycle of threshold is the cycle at which the samples reach a level of detection of a fluorescent intensity above the background and HK is the housekeeping transcript (Appendix Figure 6.2).

2.7. Western blotting

Firstly, Bradford assay (kit obtained from Pierce) was carried out to estimate the concentration of protein present in each sample. Serial dilutions of Bovine Serum Albumin (BSA) were prepared to obtain a standard curve against which the concentration of protein would be measured. In a 96- well plate, 50µl of Coomassie dye were mixed with 2µl of either standard, diluted sample or water (blank). The plate was shaken for 2-3 minutes to allow the reaction to proceed as visualized by colour changing from brown to blue. Finally, the absorbance was measured at 595nm using a plate reader and the concentrations of proteins in the samples were assessed by comparison with the standard curve.

Western blotting is a technique that allows to determine the relative amount of specific proteins namely, the expression of the protein of interest is compared to the one whose expression is not affected by experimental procedure. In particular, certain amount (µg) of protein samples (determined by Bradford assay) were diluted 1:1 in 2x loading buffer (50 ml: 2 ml mercaptoethanol, 20 ml glycerol, 4g of SDS, 25ml of Tris-HCl, 1mg bromphenol blue, dH_2O to volume) and denatured at 95°C for 5 minutes. Then the samples and a protein ladder (Bio-rad) were loaded on a 10% SDS-polyacrylamide gel (composition described in table 2.6.) and an electric field was applied to achieve protein separation on the basis of their molecular weight. The proteins were next transferred onto a methanol-preactivated Polyvinylidene difluoride (PVDF) membrane (Milipore Immobilon) by applying an electric field at 250mA for 75 minutes on ice. In order to assess the quality of the transfer, the membranes were stained with Ponceau dye for 5 minutes followed by thorough washes to visualise all the proteins. The membrane was incubated in 5% non fat milk in TBST (40.03g of NaCl, 15.76 g of Tris base, 5ml of Tween (Sigma) in 5 litres of dH_20) at room temperature for 1 hour to prevent non-specific binding of the antibodies. Primary antibodies were diluted in 1% milk/TBST as described in table 2.7 and incubated overnight at 4°C. The membranes were washed three times with TBST at room temperature, the first wash lasting 15 minutes while other two lasting 5 minutes each. Then, secondary antibodies, diluted in 1% milk/TBST, as reported in table 2.7 were incubated at room temperature for 1 hour followed by three washes with TBST as described above. The proteins of interest were detected using ECL Plus chemiluminescence detection kit (Healthcare – Amersham) and finally the membranes were exposed to X-ray films. Quantification of proteins was carried out by densitometry using Alpha Imager computer software. In particular, the integrated density of each band was acquired and the ratio between densitometric values of the experimental and housekeeping protein for each sample was performed (Raina et al, 2021, Raina et al, 2020). The relative variation in protein expression was finally calculated by comparing this ratio with the one of the control samples.

Reagents	Separating gel (2 x 0.75 mm thick gel)	Stacking gel
dH_2O	3.1 ml	1 ml
30% Acrylamide solution	2.4 ml	300 µl
Separating gel buffer / Stacking gel buffer (4x)	1.9 ml	444 µl
10% ammonium persulfate	112 µl	28 µl
TEMED	5 µl	5 µl

Table 2.6. Composition of 10% SDS-polyacrylamide gel

	Target protein	**Source**	**Dilution**	**Company**
Primary Antibody	GLE1	Rabbit	1:10000, 1:2500	Santa Cruz
	GFP	Rabbit	1:2500	Clontech, 632460
	α-tubulin	Mouse	1:2500	Calbiochem, CP06
Secondary Antibody	Anti-Rabbit IgG	Goat	1:4000	Dako, P0448
	Anti-Mouse IgG	Goat	1:10000	Bio-Rad, 170-6516

Table 2.7. Primary and Secondary antibodies used for Western Blotting

2.8. Immunocytochemistry

Immunocytochemistry is a technique that allows the visualisation of a protein taking advantage of the binding between antigen and antibody. In particular, a first antibody (primary) is used to label the protein of interest, whilst a further antibody (secondary) is usually fluorophore-labelled and recognises the primary antibody (Wakhloo et al, 2013, Pan et al, 2019, Mahajani et al, 2010).

2.8.1. Motoneuron fixing

To fix the motoneurons, the media present in the dish was removed and a rinse was performed with DPBS. After removing the DPBS, the cells were incubated with 4% Paraformaldehyde (PFA, Sigma) in DPBS at room temperature for 15-20 minutes. Motoneurons were finally rinsed and stored at 4^{o}C till the staining procedure was performed.

2.8.2. Motoneuron staining

Firstly, motoneurons were incubated with 0.3% Triton and 10% Goat serum in DPBS for 1 hour at 4^{o}C to permeabilize the cell membrane and to prevent non-specific binding of the secondary antibody. The cells were then incubated overnight at 4^{o}C with primary antibodies diluted in DPBS as described in table 2.8. To remove excess unbound antibody, the motoneurons were then rinsed 3-4 times with DPBS for 5 minutes each and next day they were incubated at 4^{o}C for 3 hours with secondary antibodies (Wakhloo et al, 2020) diluted in DPBS as described in table 2.8. Finally, the cells were rinsed as described before and the coverslips were mounted on a slide using a mounting medium optimised for fluorescent samples.

	Target protein	**Source**	**Dilution**	**Company**
Primary Antibody	GLE1	Rabbit	1:100	Santa Cruz
	α-tubulin	Mouse	1:500	Calbiochem, CP06
Secondary Antibody	Mouse IgG-CY3	Goat	1:100	Jackson, 115-165-003
	Mouse-FITC	Goat	1:200	Jackson Immunoresearch
	Rabbit A568	Goat	1:500	Invitrogen, A11036

Table 2.8. List of primary and secondary antibodies used for immunocytochemistry

2.8.3. Motoneuron Imaging

Random images of motoneurons were taken with the help of imaging software, 'Improvision Openlab 5.0.2' by Improvision Ltd and using an upright microscope 'Axioplan2' by Zeiss (Psol et al, 2021, Mahajani et al, 2021). A magnification of x20, x63 and a suitable optical filter was used to detect fluorescence.

2.9. Axonal length measurement

Axonal length measurement was done with the help of Image-J software. The longest process of each motoneuron was considered as the axon, but when there were further ramifications, the length of the longest branch was measured. In particular, segmented lines were used to track the axons and then measured.

2.10. Survival Assay

The survival of the motoneurons was also assessed by counting them before transducing with the viral vector and then on the 6^{th} day after transduction (Pietro et al, 2015, Marotta et al, 2016). Specifically, this was done by dividing the 35mm dish with 10mm rings tissue culture plates into halves by drawing a line under the ring and counting the number of half field motoneurons along the line using a 10x microscope objective.

CHAPTER 3

RESULTS

3.1. Lentiviral vector titration and validation in HEK293T cells

In order to generate tools to overexpress GLE1, lentiviral vectors were produced as described in 'Materials and Methods' section and the titres were determined by quantifying the amount of the viral protein p24 using ELISA technique. The titres listed in table 3.1, were presented as Transducing Units (TU)/ml, namely the approximate number of the functional viral particles present per ml.

Viral vectors	1st batch Titre (TU/ml)	2nd Batch Titre (TU/ml)
LV-GLE1a	8.0×10^8	2.48×10^9
LV-GLE1b	7.0×10^8	1.24×10^9

Table 3.1. Lentiviral Vector Titres for LV-GLE1a and LV-GLE1b estimated by ELISA

As the p24 measurement does not allow us to discriminate between empty viral capsid and functional virus, validation of the vectors was performed by two techniques namely, Quantitative - PCR (Q-PCR) to check the mRNA transcript and Western blotting to assess GLE1a or GLE1b protein expression after transduction. In particular, HEK293T cells were transduced with the 2nd batch of viral vectors at different MOIs and harvested 3 days after transduction for protein analysis and 6 days after transduction for total mRNA content and protein analysis.

We observed that the cells did not display any sign of toxicity, nor obvious difference in growth rate even at higher MOI of the viral vectors.

Western blotting performed after 3 days of plating, showed no GLE1 overexpression (Data not shown)

Moreover, in samples harvested 6 days post transduction, there was little over expression with LV-GLE1a virus (Figure 3.1A) but none with LV-GLE1b (Figure 3.1B).

We therefore decided to investigate whether viral transduction could increase GLE1a and GLE1b mRNA and RT-QPCR was performed as described in 'Materials and Methods'. Firstly, in agreement with previous reports (*Kendirgi et al, 2003)*, we observed that GLE1b transcript is more represented (about 20 times) than the GLE1a isoforms in untransduced cells (Data not shown).Furthermore, a dose-response increase in each specific transcript was observed, although in LV-GLE1a transduced cells the amount of overexpression of the specific transcript was about 10 times higher for each MOI than what we observed in LV-GLE1b transduced cells (Figure 3.2B).

On the basis of these results, we decided to rely on the 1st batch of LV vectors for experiments in primary motoneurons.

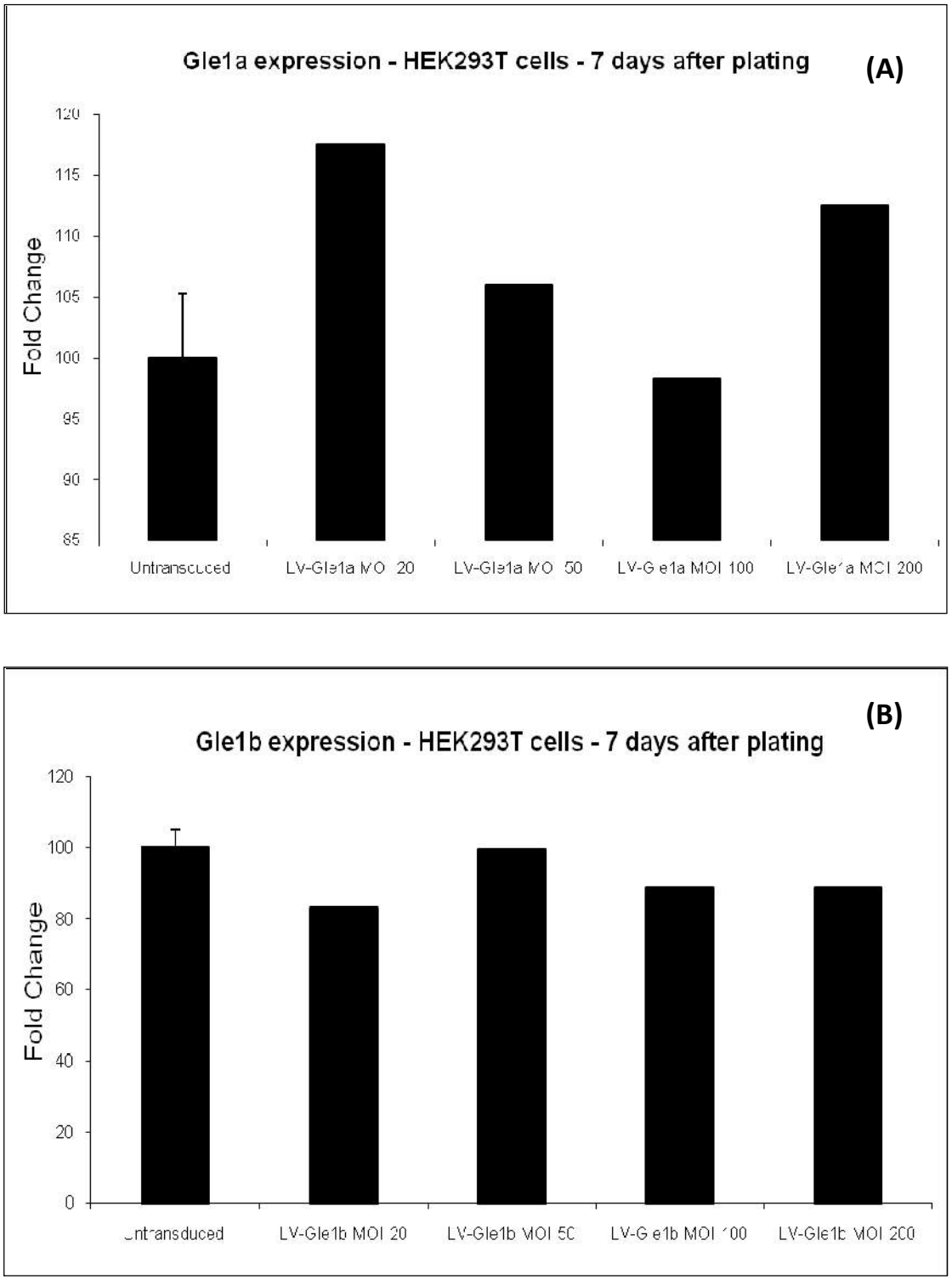
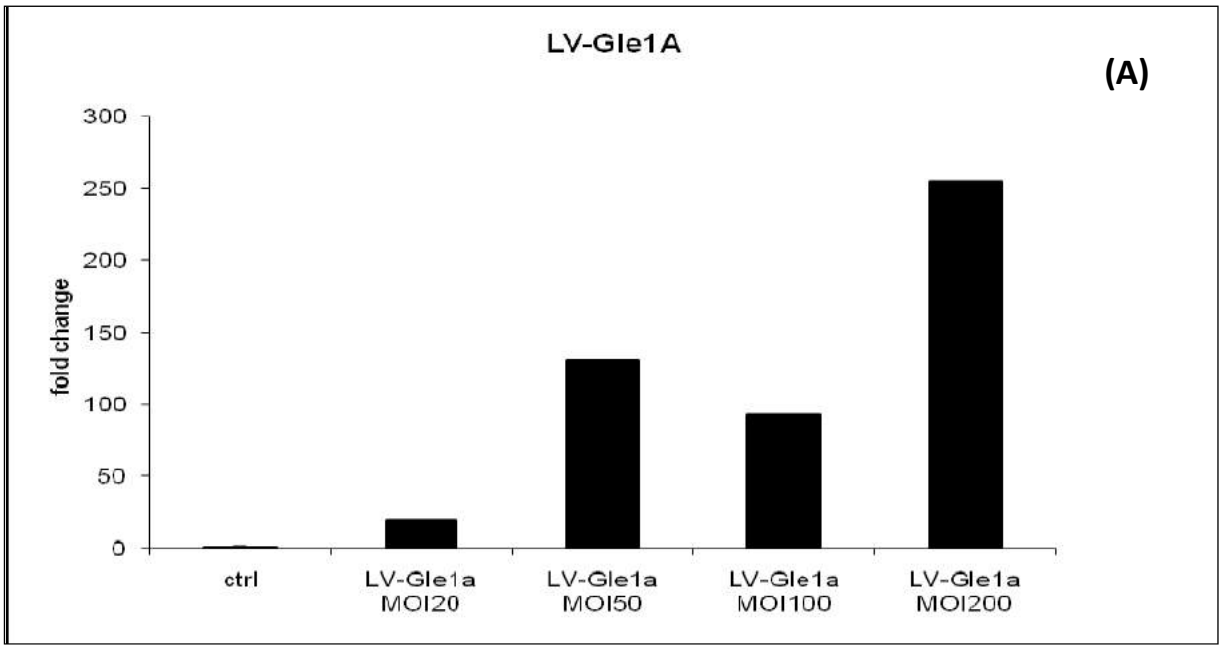


Figure 3.1. Validation of viral vectors by Western blotting. HEK293T cells were transduced with LV-GLE1a (A) or LV-GLE1b (B) with a range of MOI namely, 20, 50, 100 and 200. Untransduced cells were considered as control for this experiment.



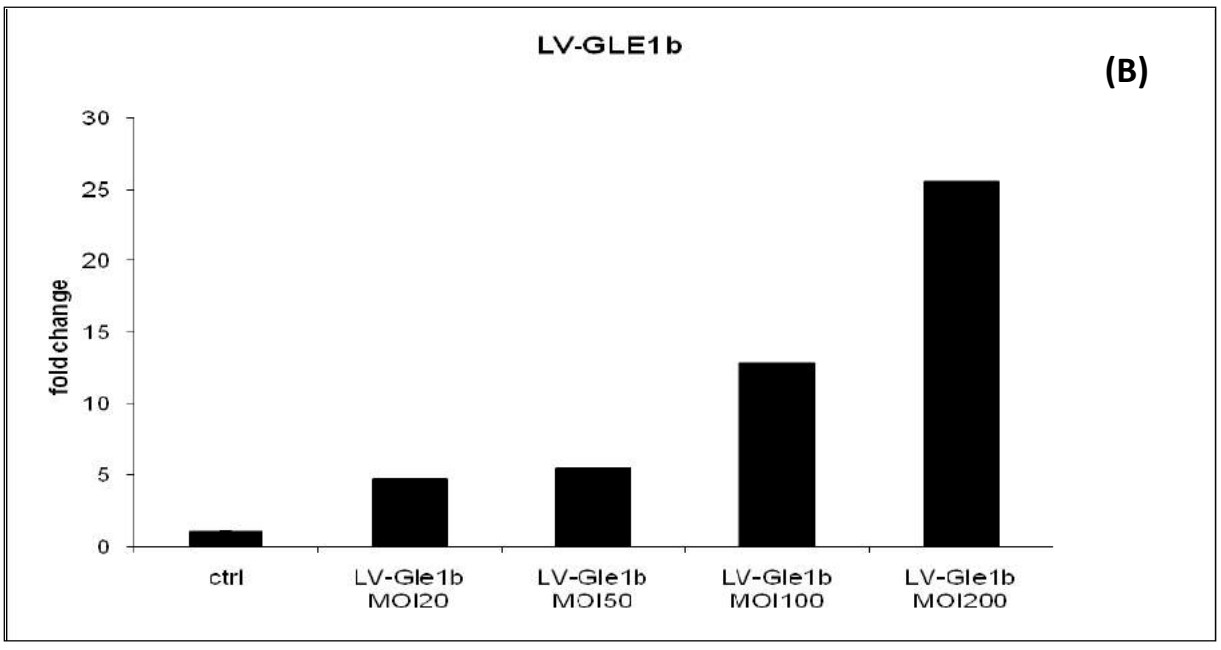


Figure 3.2. Validation of viral vectors by Q-PCR to quantify GLE1a (A) or GLE1b (B) mRNA levels in HEK293T cells transduced with different MOIs.

3.2. Lentiviral vector mediated expression of GLE1 in primary motoneurons

In order to modulate GLE1a or GLE1b expression, primary motoneurons were transduced with specific lentiviruses and protein expression was validated by immunocytochemistry and Western blotting.

Immunocytochemistry was preformed to visually assess GLE1 expression 6 days after transduction with LV-GLE1a or LV-GLE1b as compared to untransduced motoneurons. As described in 'Materials and Methods', we performed a double immunostaining using anti - GLE1 and anti - α-tubulin, a marker of the cytoskeleton of the motoneurons along with Hoechst staining to visualise motoneuron nuclei.

In untreated motoneurons, we observed a diffuse GLE1 staining in the cell body and in the axon (Figure 3.3, first two rows). Conversely, increased amount of green colour can be seen in transduced SMA motoneurons as compared to untransduced motoneurons suggesting that there is an over expression of GLE1a or GLE1b after addition of the virus (Figure 3.3, last two rows).

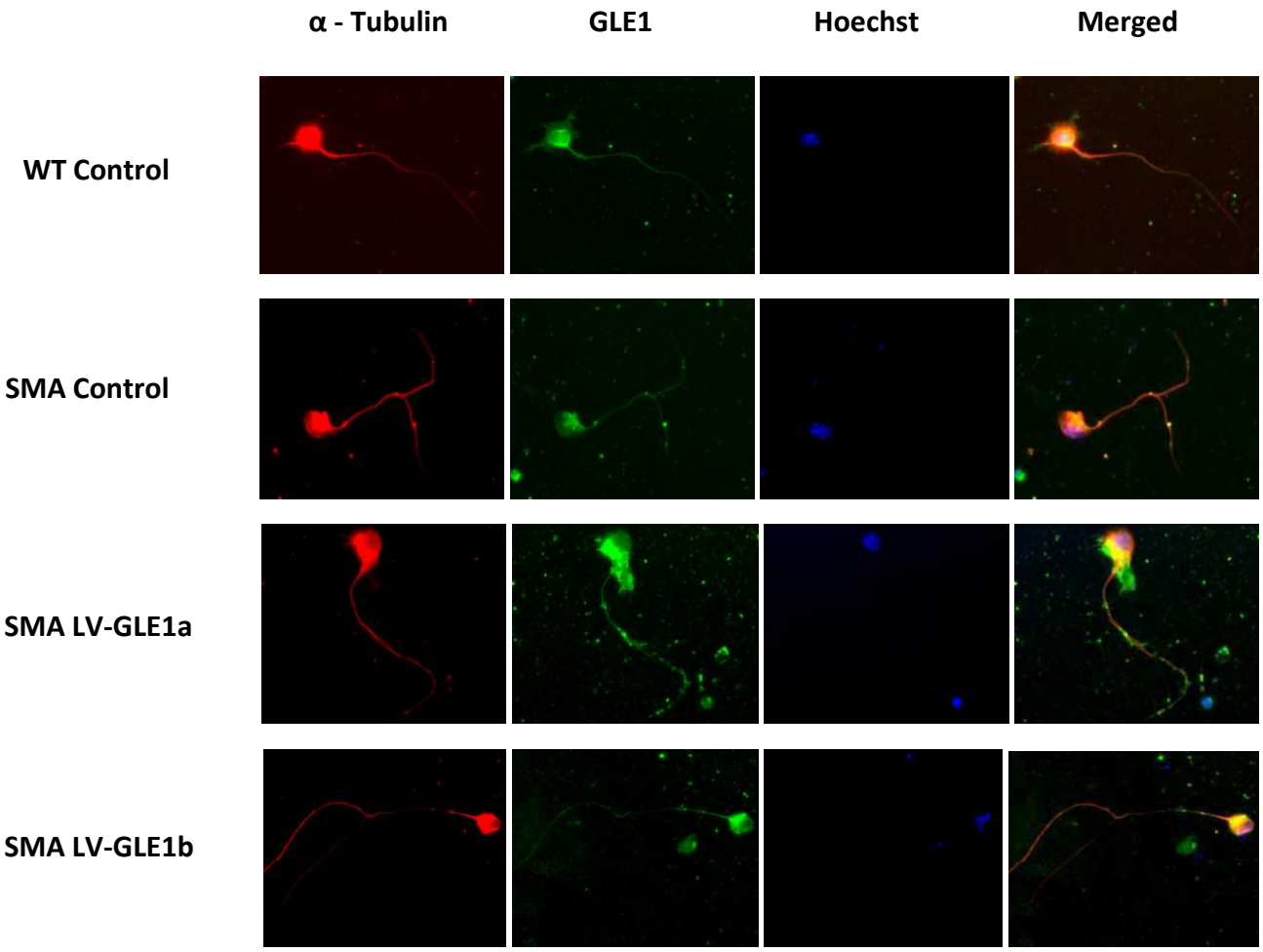


Figure 3.3. Images showing wild type and SMA ($Smn^{-/-}$) motoneurons transduced with LV-GLE1a or LV-GLE1b and stained with antibodies specific to either GLE1a or GLE1b.

As immunocytochemistry does not allow a quantitative assessment of the protein expression, we decided to check GLE1 expression by Western blotting. Briefly, motoneurons were transduced with MOI 50 and MOI 100 of LV-GLE1a and LV-GLE1b and harvested 6 days post transduction and GLE1 expression in those cells were compared to the endogenous levels in untransduced motoneurons.

In Figure 3.4A, the upper row shows the GLE1 bands obtained and the lower row depicts α -tubulin bands. Densitometric analysis revealed an increase in GLE1 protein level when motoneurons were transduced with LV-GLE1a but not with LV-GLE1b. GLE1a level seems to be higher for MOI50 than for MOI100 (Figure 3.4B).

However, a good commercially available antibody targeting GLE1 does not exist. The one we use targets a C-terminal sequence of human GLE1 common to both isoforms and highly homologous to the mouse protein. Nevertheless, when we were establishing the best experimental conditions for Western blotting, we struggled to find a dilution that was not giving us non specific bands, thus raising doubts about the purity of our antibody. On the basis of this, we suspect that in motoneuron extracts, where GLE1 expression seems to be particularly high, our antibody might not provide us enough sensitivity to detect smaller increase in GLE1 expression such as those trigger by LV-GLE1b transduction (see figure3.2)

Therefore, based on these results, we decided to use only LV-GLE1a in further experiments.

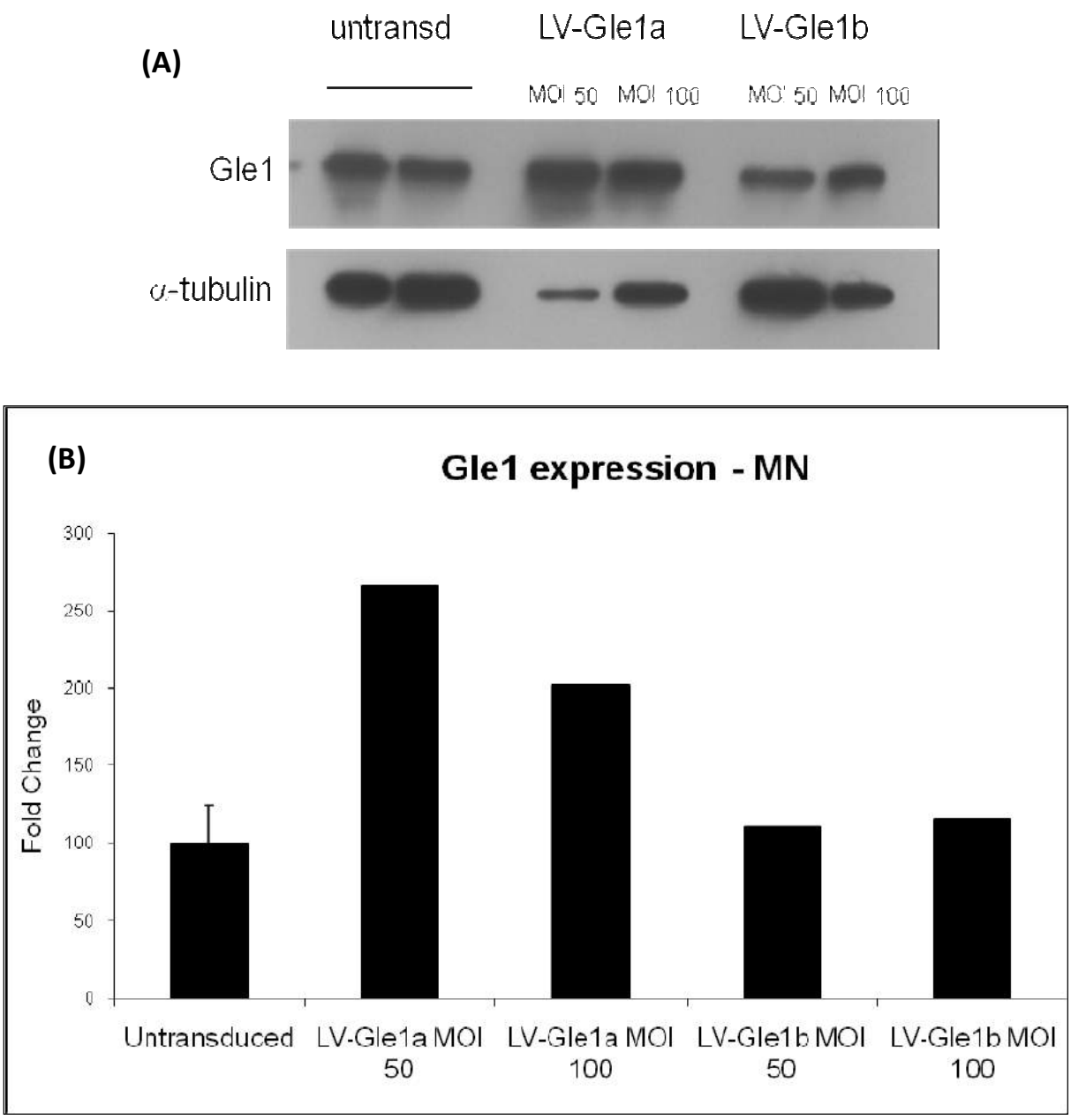


Figure 3.4. Western blotting analysis for GLE1 in purified motoneurons (A) Representative film showing GLE1/α-tubulin expression in motoneurons transduced with either LV-GLE1a or LV-GLE1b at various MOIs. (B) Band intensity was determined by densitometric analysis showing increased expression of GLE1a but not GLE1b as compared to untransduced motoneurons.

3.3. Impact of GLE1 overexpression on motoneuron survival

In order to investigate the effects on cell viability of GLE1 overexpression a survival assay was performed by counting the motoneurons once before transduction (day 0) and once after 6 days of transduction i.e. 7 days after plating (day 7).

Unfortunately, contamination was observed in some of the samples therefore, KO untransduced cells could not be counted on day 7. Moreover, this assay was performed only once, the results should be considered only indicative, although there is no indication of a trophic activity of GLE1 in primary motoneurons, as no sustained survival was observed in GLE1 over expressing motoneurons over untransduced ones (Figure 3.5). This data set is obtained using only one *SMN*$^{-/-}$ embryo (n=1), whereas to obtain any significant proof it has to be repeated to at least n=3.

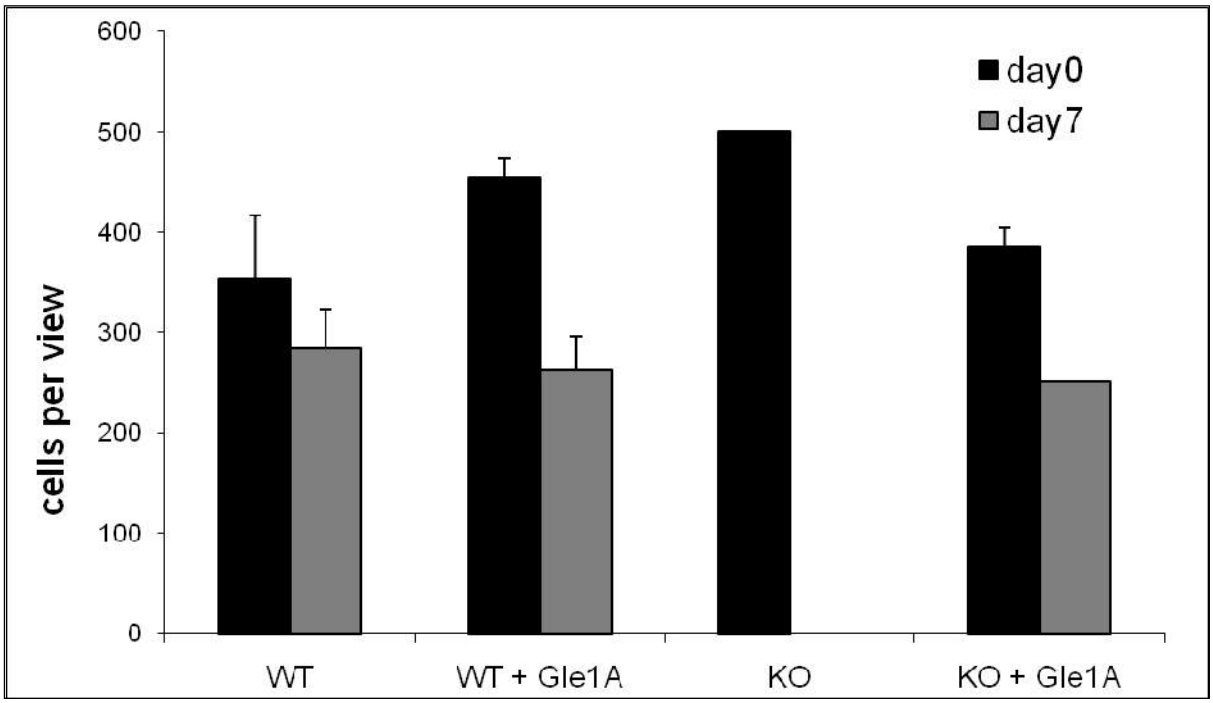


Figure 3.5. Survival assay for primary motoneurons. Motoneurons were counted on Day 0 and Day 7 for Wild Type ($Smn^{+/+}$) untransduced and transduced with GLE1a, Knock out ($Smn^{-/-}$) untransduced and transduced with GLE1a (n = 1).

3.4. GLE1 overexpression rescues axonal defects in SMA motoneurons

From the literature, we knew that cultured motoneurons purified from a severe model of SMA displayed reduced axonal length *(Jablonka et al, 2004)*. We therefore aimed at assessing whether such abnormality could be observed also in motoneurons purified from SMNΔ7 mice.

To achieve this, random motoneurons were selected and pictures taken for axonal length measurement which was performed as described in 'Materials and Methods'. Around 40 motoneurons for each group/treatment obtained from 4 different SMNΔ7 embryos (n=4) were included in this analysis.

Representative examples of the motoneurons used for axonal length measurement from various groups are shown in figure 3.6.

Firstly, we observed that the difference of lengths of axons between WT and SMA motoneurons is significant ($p<0.001$ with one way ANOVA and Bonferroni test) (figure 3.7) as the mean of length of axons for SMA nearly half as compared to WT, a finding that is consistent with the published data *(Jablonka et al, 2004)*. Interestingly, there is a statistically significant increase in the length of axons of SMA motoneurons after transducing them with LV-GLE1a. The average length of axons of SMA motoneurons after transducing is almost equal to the length of the axons of WT untransduced motoneurons. This effect on axonal growth seems to be specific to SMA motoneurons as there is no significant different between WT untransduced and WT transduced with LV-GLE1a (Figure 3.7).

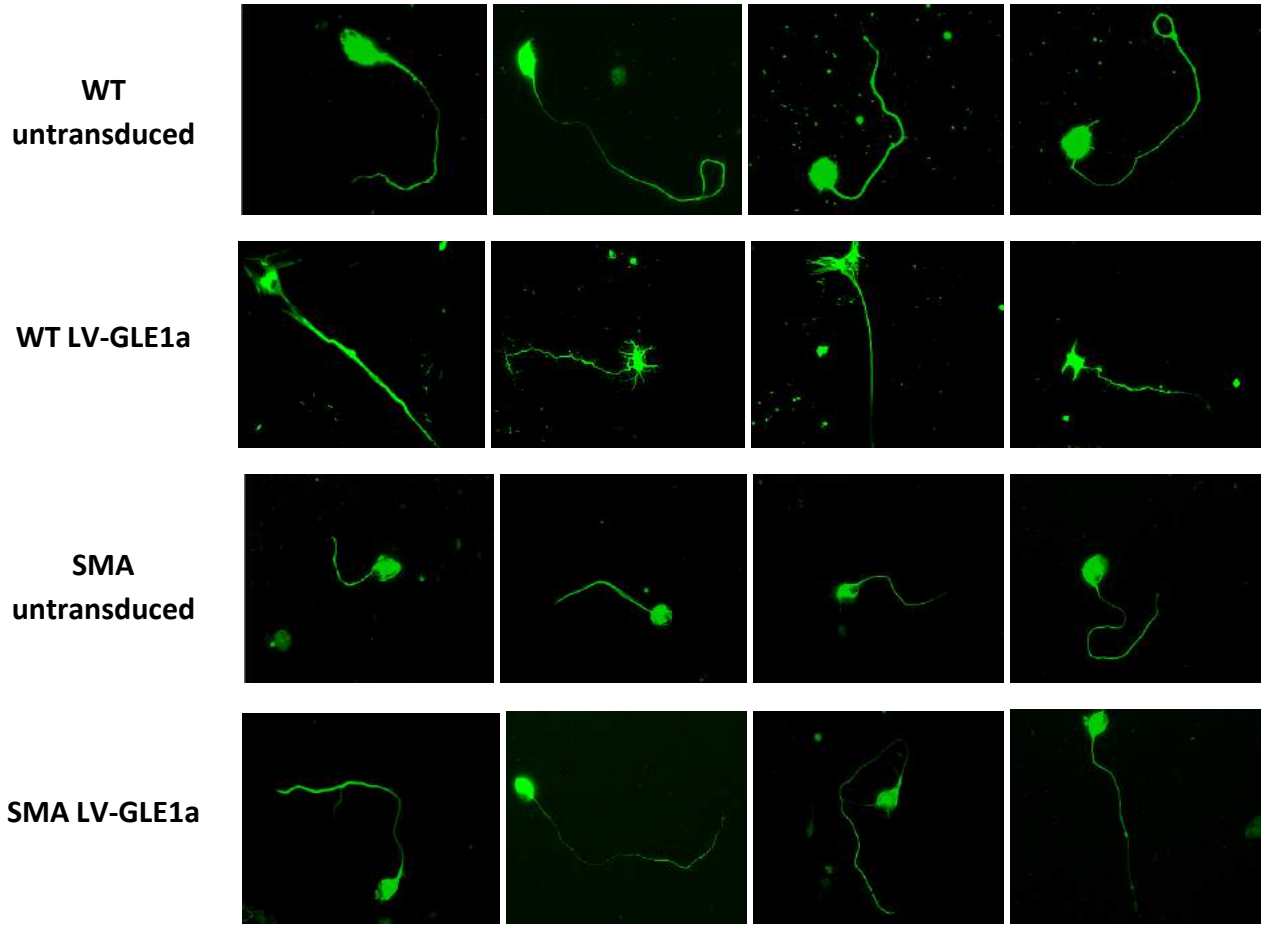


Figure 3.6. Axonal length measurement representing examples of cultured wt or *Smn*$^{-/-}$ (SMA) motoneurons 6 days post treatment with the indicated lentiviral vectors.

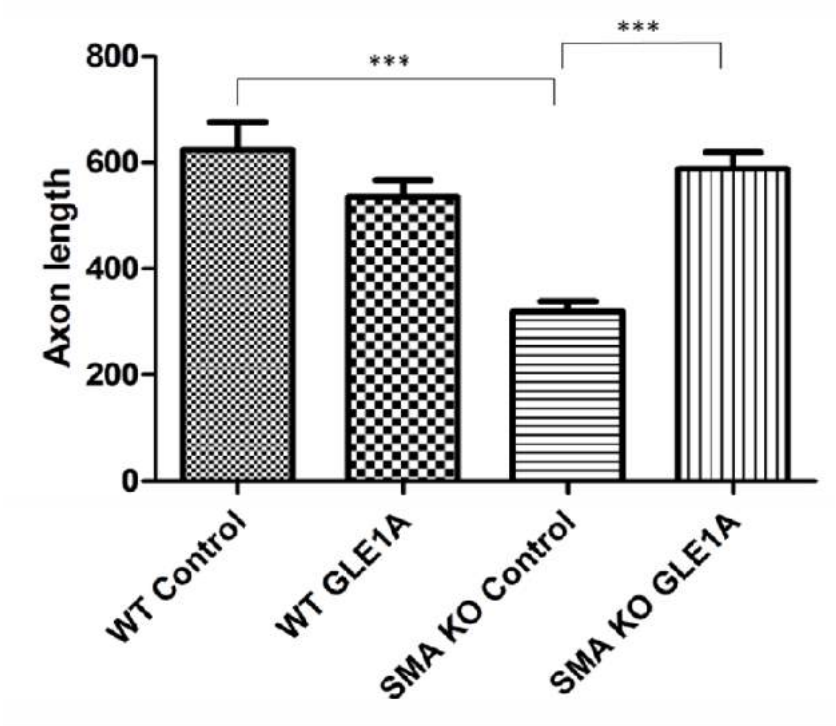


Figure 3.7. Axonal length (μm) measurement representing the average length of their axons of groups/treatment. α-tubulin immunostaining was used to visualize the cytoskeleton. (n = 4, *** $p<0.001$ one way ANOVA and Bonferroni test)

CHAPTER 4

DISCUSSIONS

In this project, we show that (1) Smn deficient motoneurons from a mouse model of SMA display reduced axonal elongation and that (2) such axonal defects can be specifically rescued by GLE1a overexpression, as confirmed by Western blotting and immunocytochemistry.

4.1. SMA and LCCS

Spinal Muscular Atrophy, an autosomal recessive disorder, is one of the leading genetic disorders affecting children with an incidence rate of nearly 1 in 6000 births and with the onset of disease generally ranging from 2 months of age to adulthood. However, foetuses dying before 32nd gestation week and displaying severe motoneuron dysfunction were initially diagnosed as SMA severe cases. Nowadays, those foetuses are correctly diagnosed with Lethal Congenital Contracture Syndrome (LCCS) *(Nousiainen et al, 2008).* LCCS and SMA were initially thought to be caused by mutation or loss of the same gene(s) because both disorders exhibit similar phenotypes.

Despite those similarities, linkage analysis demonstrated that the LCCS and SMA were different; thereby suggesting different gene(s) are responsible for these two disorders *(Vuopala et al, 1995)*. SMA, a monogenic disorder, is caused by the loss of *SMN1* gene resulting in the scarcity of SMN protein required in mRNA splicing. Presence of variable number of copies of *SMN2* gene partially contributes in compensating the loss of *SMN1* gene as *SMN2* is capable of producing only 10% functional SMN protein. Its transcript undergoes alternative splicing generating mRNA lacking exon 7 and this is translated in a C-terminal abnormal SMN protein that is rapidly degraded. In keeping with this, the severity of the disorder is inversely proportional to the number of copies of *SMN2* gene. Normally, 0-3 copies are reported in humans, whereas a case with 8 copies of *SMN2* gene has also

been reported *(Vitali et al, 1999; Wilton, 2009)*. On the other hand, LCCS was first observed in a Finnish family and is categorised into three sub-types based on the symptoms observed. It has been identified that deficiency of GLE1 is responsible for LCCS1 whereas mutations in *ERBB3* gene and *PIPK51C* gene lead to LCCS2 and LCCS3 respectively. No mutations or deletions were seen in *SMN1* gene thus confirming that SMA and LCCS are genetically unrelated disorders despite the clinical similarities *(Nousiainen et al, 2008; Narkis et al, 2007a; Narkis et al, 2007b).*

Interestingly, recent findings in zebrafish (*Danio rerio*) added further evidence of a common detrimental phenotype triggered by depletion of either Smn or Gle1. Firstly, morpholino antisense nucleotide (MO) targeting *Smn* were injected shortly after fertilization to assess the impact of Smn deficiency in early embryological development and defects in axonal pathfinding as well as intensive branching were observed *(McWhorter et al, 2003).* Along with these observations, shorter axons were also reported *(McWhorter et al, 2003).* Similarly, injection of MO targeting *Gle1* in zebrafish embryos caused axonal defects in a dose-dependent way suggesting Gle1 depletion affects axonal growth *(Seytanoglu et al, unpublished data)*. Moreover, preliminary observations of transgenic zebrafish whose G*le1* gene has been disrupted by insertional mutation suggested that Gle1 deficiency leads to reduced mobility, developmental abnormalities and death within few days post fertilization *(Amsterdam A et al, 2004 and Seytanoglu et al, unpublished data)*

Since lack of either SMN or GLE1 seems to equally affect motoneuron viability, it is important to investigate the function of those proteins. In particular, SMN is found ubiquitously and is involved in the formation of small nuclear ribonucleoprotein assembly, which is responsible for splice sites recognition and removal of introns from pre-mRNA. High level of SMN protein has been reported in motoneurons obtained in the spinal cord *(Burghes and Beattie, 2009).*

On the other hand, GLE1 best characterised function is facilitating the export of mRNA from the nucleus to the cytoplasm for translation into protein *(Hurt and Silver, 2008).* GLE1 therefore interacts with several proteins involved in this cellular process. Furthermore, its role seems critical especially for motoneurons (Nousiainen et al, 2008) in which we demonstrated high expression by immunocytochemistry and Western blotting.

Interestingly, as SMN and GLE1 proteins are involved in splicing and export of mRNA respectively, we might consider them as acting on two different steps or phases of the same pathway that includes transcription, pre-mRNA splicing, mRNA export to cytoplasm and translation into a protein. Therefore, either SMN or GLE1 deficiency might lead to reduced translation of protein specifically important for motoneurons thus leading to similar defects.

As a matter of fact, it is still unclear why SMN or GLE1 deficiency makes the motoneurons specifically vulnerable to cell death, even though these proteins are expressed ubiquitously. More investigations have been preformed aiming at elucidating a specific role for SMN in motoneurons. Firstly, when SMN expression was assessed by immunohistochemistry in rat spinal cord at different ages, it was earlier observed that there was a progressive down regulation of the protein in non-neuronal tissues from pre-natal to adulthood, but not in motoneurons *(Pagliardini et al, 2000)*. In particular, it was observed that there was cytoplasmic as well as nuclear localisation of SMN in neurons, suggesting that different isoforms of SMN protein may be distributed in various cellular compartments *(Battaglia et al, 1997)* and interestingly, SMN protein was seen to be localised in the axons of the motoneuron hinting at its involvement in axonal transport *(Pagliardini et al, 2000).* More recently, SMN along with heterogeneous nuclear ribonucleoprotein R (hnRNP-R) was suggested to be essential for the transport of β-actin mRNA along the axon thus contributing to axon initiation, growth, guidance and branching *(Rossoll et al, 2003).* This importance of the interaction was further confirmed when knockdown of hnRNP in zebrafish and mouse model mimicked the detrimental

reduction in axonal elongation observed in SMA models *(Glinka et al, 2010).* Conversely, nothing is known about the physiological function of GLE1 in motoneurons and the mechanisms linking *GLE1* mutation to motoneuron loss in LCCS have not been investigated yet.

4.2 Modulation of GLE1 expression in motoneurons

In order to gain more insight about the role of GLE1 in motoneurons, we first produced lentiviruses to drive the expression of either GLE1a or GLE1b and we aimed at confirming that our vectors were functional by different techniques. As the exact function of these proteins is still unknown, we decided to investigate the effect of either isoforms and therefore we tried to validate both the lentiviral vectors encoding GLE1a or GLE1b. To achieve this aim we transduced a highly permissible cell line, HEK293T and we assessed the expression of our gene of interest at mRNA and protein level by RT-QPCR and Western blotting, respectively. While we could demonstrate increased transcription from both our constructs, we only observed GLE1 overexpression upon transduction with LV-GLE1a. Furthermore, we also transduced purified motoneurons, our final experimental model, but disappointingly, no overexpression was observed after transducing the motoneurons with LV-GLE1b. We therefore, decided to focus our efforts mainly on the impact of GLE1a modulation.

4.3. Effects of GLE1 overexpression on motoneuron survival

Although SMA is a neurodegenerative disease, the exact mechanisms of cell death are not fully understood. Furthermore, histopathological studies on spinal cord from different cases of SMA

provide controversial results about the timing and the relevance of motoneuron over synaptic loss *(Soler-Botija et al, 2002; Ito et al, 2010).*

In SMNΔ7 mice, however, significant motoneuron loss precedes the end stages of the disease *(Le et al, 2005).*

To investigate SMA motoneuron viability, we designed an experiment to check (1) whether SMN deficiency would reduce motoneuron survival in cells purified from SMNΔ7 mice and (2) whether over expression of GLE1a could be neurotrophic. Unfortunately, we could not achieve a conclusive result, but as a preliminary observation our experiment does not support a neurotrophic role for GLE1. Due to the lack of time, the experiment could not be repeated, but it would be interesting to check whether GLE1 overexpression affects the survival of SMA motoneurons compared to the untransduced controls.

4.4. GLE1 rescues morphological abnormalities in SMN-deficient motoneurons

When motoneurons from a mouse model of severe SMA (*$Smn^{-/-}$; $SMN2^{+/+}$*) were purified and their axonal length was measured, it was seen that there was a significant reduction as compared to the wild type motoneurons *(Jablonka et al, 2004).* Consistently, preliminary data obtained by Rengina Marketou demonstrated reduced axonal growth in cultured motoneurons depleted of SMN using shRNA silencing technique. Here, we addressed this issue in purified motoneurons from SMNΔ7 mice model and for the first time we demonstrate significant reduction of axonal length. Specifically, significant reduction (about 50%) in axonal elongation in SMA (*$Smn^{-/-}$; $SMN2^{+/+}$;* SMNΔ$7^{+/+}$) versus Wild Type (*$Smn^{+/+}$; $SMN2^{+/+}$;* SMNΔ$7^{+/+}$) cells is reported. On the basis of this evidence, we therefore speculate that shortening of axons is an intrinsic feature of SMN deficiency in motoneurons.

In order to assess whether GLE1 overexpression could rescue SMN-induced axonal defects, we transduced SMA and Wild Type motoneurons with LV-GLE1a. Interestingly, transduced cells showed a significant increase in the axonal length as compared to untransduced SMA motoneurons, thus restoring the morphological deficit caused by Smn deficiency. Furthermore, the axonal length of these motoneurons was not affected by LV-GLE1a transduction, thus suggesting that GLE1a overexpression alone was responsible for compensating the specific impact of SMN deficiency on motoneuron morphology. However, further experiments are necessary to confirm this finding.

4.5. Viral vectors as promising tools for gene therapy

Our *in vitro* results suggest that GLE1 over expression might rescue SMA specific deficit in axonal growth thereby supporting the interest in investigating GLE1 as gene modifier in SMA mouse models. So far, the best therapeutic option seems to be SMN replacement by viral mediated gene therapy. Early experiments were performed on SMNΔ7 mice that were injected intramuscularly with a lentiviral vector expressing human SMN and capable of retrograde transport to the spinal cord motoneurons. This treatment increased the survival of SMA mice by an average of 5 days over untreated SMA mice, thus providing the first proof of principle for SMN replacement *(Azzouz et al, 2004b).* More recently, we (Valori et al, 2010) and others (Foust et al, 2010) reported a dramatic increase in survival of SMNΔ7 mice when SMN replacement was achieved though intravenous administration of self-complementary adeno-associated virus serotype 9 (scAAV9). The potential for this treatment to be translated into a clinical trial was supported by studies performed in non-human primates. In particular, transduction of spinal cord motoneurons was seen when scAAV9 was injected in newborn monkey (*cynomolgus macaque*) thereby confirming that this viral vector can

also cross the blood brain barrier in primates *(Foust et al, 2010).* Moreover, intra spinal administration of either scAAV8 or AAV8 greatly extended SMA mice survival *(Passini et al, 2010).* However, AAV-based SMN replacement strategies are still to be tested in human trials to check their efficiency for this devastating disorder.

Given the promising outcome of our *in vitro* study it would be interesting to test the effect of GLE1 overexpression *in vivo* in SMNΔ7 mice. Therefore as a next step for this project we might generate viral vectors to drive GLE1a expression for *in vivo* efficacy studies in SMA mouse models. In conclusion, based on results obtained we propose GLE1 as a potential neuroprotective target for Spinal Muscular Atrophy.

CHAPTER 5

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CHAPTER 6

APPENDIX

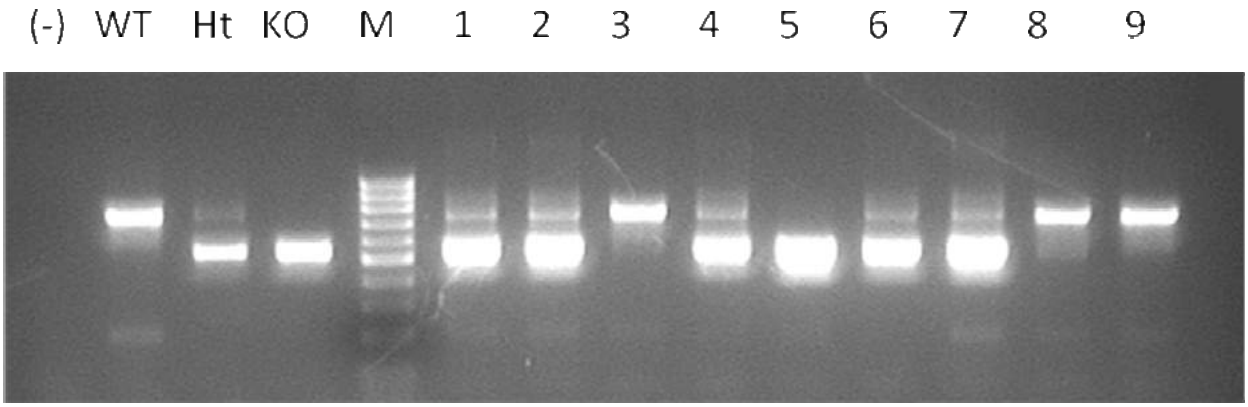

Figure 6.1. Example of genotyping. PCR to detect *Smn* target mutation was performed and the amplicons were separated on an agarose gel. The first lane is (-) is the negative control, whilst the three following are three positive control, as indicated. After the DNA ladder, we loaded the experimental samples. Out of 9 embryos obtained, 3 were found to be Wild Type ($Smn^{+/+}$) (No.3, 8, 9), whereas 5 were found to be Heterozygous ($Smn^{+/-}$) (No. 1, 2, 4, 6, 7) and only one Knock Out was seen ($Smn^{-/-}$) (No. 5)

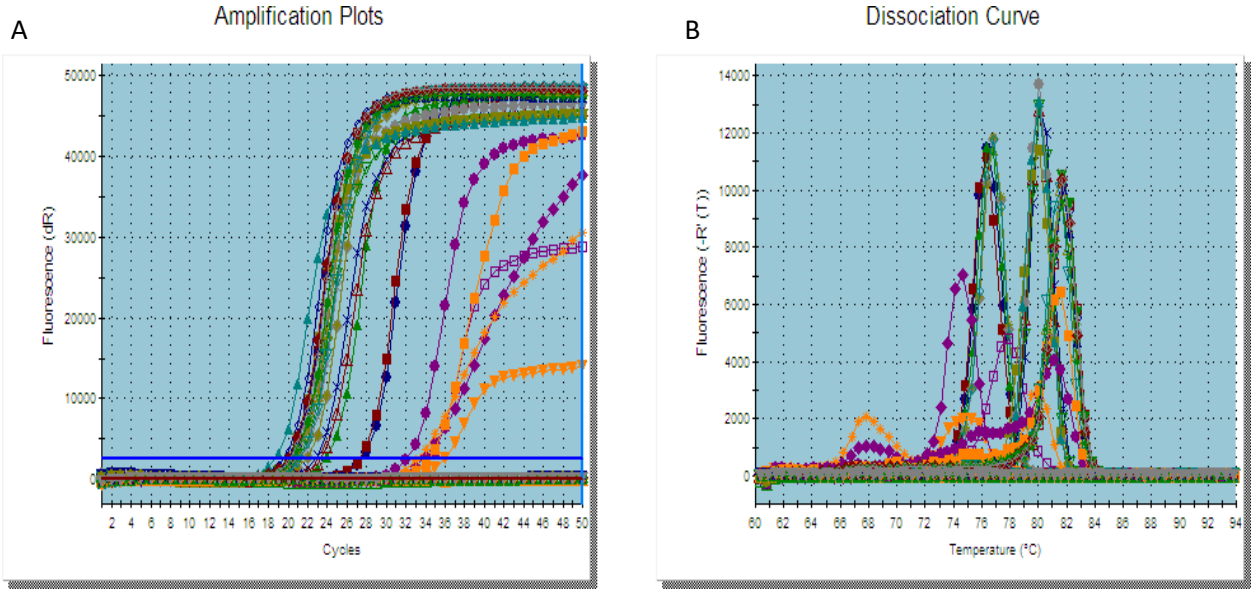

Figure 6.2. Amplification and dissociation curves for ΔΔCt method (A) Amplification plots showing fluorescence (Y-axis) against number of cycles (X-axis). The software calculates the Ct (Cycle of threshold, depicted as horizontal blue line) in which the fluorescence associate to a specific sample becomes significant (B) Fluorescence (Y-axis) against temperature (X-axis) is represented. This parameter allows establishing the number of amplicons in each sample. For accurate results, only 1 species should be amplified.